Relationship of Steroid Receptor, Cell Kinetics, and Clinical Status in Patients with Breast Cancer

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ABSTRACT

The fractions of cells in the different phases of the cell cycle were determined by flow cytometry in 70 human breast tumors and six human benign breast tissues. This procedure showed that 44% of the tumors and none of the benign tissues were aneuploid as determined by mixing experiments using normal peripheral blood as a standard for DNA content per nucleus. The mean percent S-phase fraction (% S) values ± S.D. for benign and malignant tissues were 6.9 ± 1.6 and 13.7 ± 6.5, respectively. By our procedure, aneuploid tumors seem to have significantly higher mean % S value than do diploid tumors. Breast cancer tissue which contained steroid receptors had a mean % S value of 11.3, while those tumors which had neither the estrogen nor progesterone receptors had a mean % S value of 17.1 (p < 0.01). The estrogen receptor status had a better inverse relationship to the cell kinetic data than did the progesterone receptor status. The use of molecular forms of the steroid receptor was of some assistance in improving the inverse relationship between cell kinetics and steroid receptor status. A trend was observed between lack of steroid receptors and higher probability of the tumor being aneuploid. From the limited clinical data, there was no relationship between cell kinetic and aneuploid data with respect to nodal status, metastatic disease, and menopausal status. The possible use of these data is discussed.

INTRODUCTION

Breast cancer has now become the most common cancer in the United States to afflict women. The incidence continues to rise, and it is estimated that there will be 109,000 new cases and 36,000 deaths due to breast cancer in 1980 (5). In spite of major advances in the chemotherapy and the hormonal therapy of this disease during the last decade, the death rate has remained constant, and cure is rare after development of distant metastasis.

Recent advances in research have increased our understanding of this illness. The discovery and characterization of steroid hormone receptors have enabled the clinician to more accurately predict the response to hormonal therapy in patients with recurrent or metastatic disease (17, 24). In addition, the recent gains in hormonal therapy, including the development and successful use of antiestrogen compounds, medical adrenalectomy, and improved surgical procedures for endocrine ablation, have been noteworthy. Several studies have indicated that patients with steroid receptor-positive tumors have a more favorable prognosis and a more indolent course (8, 13, 22); recently, however, other investigators have challenged this position (10). Studies have also appeared suggesting that ER\(^3\) may be related to response rate and duration in patients receiving chemotherapy (2). Initial work indicated that patients with steroid receptor-containing tumors have a lower response rate to chemotherapy than do those whose tumors were receptor-negative (2). Studies by other investigators reveal contradictory results (12), and recently, retrospective analyses have indicated that receptor content is probably not related to response rate (10). Other studies in these areas will be needed to determine the role of receptors in predicting clinical course.

Cell kinetic studies have given us a better understanding of the biology of breast cancer. Several investigators reported that tritiated thymidine uptake was inversely correlated with steroid receptor activity (20, 27). Meyer and Lee (22) have also shown that tritiated thymidine uptake can be related to the degree of nuclear differentiation.

The development and use of flow cytometry to determine cell cycle kinetics has been a major advance in tumor biology (21). Previous methods of cell kinetic analysis using tritiated thymidine labeling methods have been cumbersome, expensive, and time consuming. Flow cytometry enables the investigator to assay cell cycle parameters precisely and rapidly, since small amounts of tissue (50 to 60 mg) with as few as 10\(^5\) cells can give an accurate DNA histogram. Flow cytometry also has the advantage of producing precise data analysis when compared to the tritiated thymidine uptake studies, which require subjective assessment in the actual counting procedure. In addition, flow cytometry offers the investigator the ability to sort cells according to DNA content. An excellent review describing the methodology and application of flow cytometry has recently been published (19). The disadvantages of flow cytometry measurements of cell cycle kinetics are lack of histological identification of individual cells analyzed and sampling selectivity of cell population from tumor tissue. The present study was designed to determine the relationship of steroid receptor content to cell kinetics as defined by flow cytometry studies. It also includes limited clinical data which were compared with cell kinetics and steroid receptor activity.

MATERIALS AND METHODS

Steroid Receptor Assay. Primary and metastatic breast cancer tissues were received between September 1978 and February 1980 from the North Carolina Baptist Hospital, Gaston Memorial Hospital (Gastonia, N. C.), and Cleveland Memorial Hospital (Shelby, N. C.).

1 Supported by NIH Grant CA26359-01 and the Oncology Research Center-United Fund Intramural grant. Presented at the American Association for Cancer Research, May 1980, San Diego, Calif. (23).
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3 The abbreviations used are: ER, estrogen receptor; % S, percentage of S-phase fraction; PI, proliferative index; PR, progesterone receptor.

Received January 19, 1981; accepted June 8, 1981.

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Tissue samples were frozen immediately after surgery and were stored at -85°C. Approximately 0.5 to 1.0 g of tissue was homogenized, and the cytosol was prepared as described previously (15). The receptor binding activities were determined either by sucrose density gradient assay (85% of patients) or the dextran-coated charcoal assay (15% of patients). The methods used for these assays have been reported elsewhere and utilized a computer program (15).

**Preparation and Staining of Nuclei.** The preparation of the nuclear fraction of the tissue sample for use in flow cytometry was performed as follows. The frozen tissue was removed, cut into small pieces, and added to Buffer A (0.5 M sucrose: 1.5 mM MgCl2: 40 mM Tris, pH 7.4). The ratio of tissue to Buffer A was approximately 1:20. This tissue was then homogenized with a 4- to 5-sec burst of the Polytron (Brinkmann Instruments, Inc., Westbury, N. Y.) at very slow speeds and was continued until most of the solid tissue was completely eliminated. This suspension was then successively passed through 40-, and 100-, and 200-wire mesh strainers to remove connective and nonhomogenized tissue. The nuclear suspension solution was then placed in an HS-4 rotor (Ivan Sorvall, Inc., Newtown, Conn.) and centrifuged for 10 min at 100 rpm (192 x g), and then the pellet was concentrated into one test tube and washed once by centrifugation. After the nuclear pellet was suspended in 1 to 2 ml of Buffer A, the number of nuclei per ml was determined in an electronic counter (Model ZB Coulter Counter; Hialeah, Fla.). A nuclear suspension of 1 x 10^6 nuclei/ml was made with a propidium iodide staining solution as described by Krishan (14). This staining solution contains RNase (3 units/ml) for removal of the RNA-propidium iodide fluorescent interaction and 0.1% Nonidet P-40 to remove the cytoplasmic debris from the prepared nuclei (14). After 10 min of incubation at 0°C, the stained nuclei were passed through a 70-µm-pore-size mesh for removal of any further debris. The stained nuclei were examined prior to flow cytometry to check for adequate dispersal of nuclei. This stained solution was then analyzed by the Coulter TPS-1 fluorescent cell sorter. In several patients, the DNA histograms were obtained from fresh and frozen specimens of the same tumor. No differences were noted.

**Analyses of DNA Histograms.** The cells were excited at 488 nm using an American Standard laser, and the fluorescence was analyzed with the green photomultiplier which included the various necessary filters for removing laser excitation and nonfluorescent light. The analysis of the DNA histogram was continuously formed until one channel reached a maximum of 10,000 cells. The total amount of cells analyzed varied depending on the DNA histogram profile.

A representative sample of a DNA histogram done by the above procedure on various tumors and on normal tissue is shown in Chart 1. Chart 1A represents the DNA histogram obtained from normal peripheral blood leukocytes. One sees a predominant peak at the 2n region (diploid) which corresponds to the cells in the G1 (or G0) phase of the cell cycle. There are very few nuclei which contain greater fluorescence (i.e., DNA content) than this peak, indicating that there are few to no DNA-synthesizing cells (S phase) or cells ready for division (G2). The nuclei in the G2 and/or mitotic (M) phases would have a normal distribution of fluorescence twice that of the G1 nuclei, but one cannot analyze the M-phase cells in the DNA histogram by this method. S-phase nuclei would have fluorescence between these 2 peaks.

A DNA histogram of a benign fibrocystic tumor is shown in Chart 1B. One observes that the predominant type of cell in this tissue is also a resting cell in the G1 phase of the cell cycle. There are small populations of cells in the S and G2 phases. In Chart 1C, one observes a DNA histogram obtained from breast cancer tissue. There are 2 dominant peaks which correspond to the G1-phase of diploid cells and the G2 phase of tumor cells that contain an increased DNA content (aneuploid). In order to determine which peaks were aneuploid or diploid, a mixing experiment as described by Barlogie et al. (4) was performed and is shown in Chart 1D. With this method, normal DNA-containing stained nuclei from peripheral blood cells (Chart 1A) were mixed with the stained tumor nuclei. The normal nuclei enhanced the diploid peak and diminished the aneuploid population. A large concentration of cells in the S and G2 phases for the aneuploid cells is also seen in Chart 1C, which is different from Chart 1A and B.

DNA histograms were recorded on a Texas Instruments Silent 700 (Texas Instruments, Houston, Texas), and the data were stored on magnetic tapes. The analysis of the DNA histogram data was performed using a program developed by Fried et al. (7), which determines the percentage of cells in each phase after curve-fitting the raw data. In all the tumors analyzed, the fit of the raw data corresponded with the fit of the curves defined by the Fried program. PI in this study is defined by the equation

\[
PI = \frac{S + G2}{G1 + S + G2} \times 100
\]

In the case of aneuploid tumors or histograms showing 2 separate G1 peaks, the cell kinetics data were determined only for the aneuploid subpopulations of the tumor. Only DNA histograms on tumor tissue with clear separation of diploid and aneuploid peaks were used in this study.

**Pathological Evaluation.** All tissue samples were histologically reviewed to confirm the presence of malignant tissue. Portions of the sample used for steroid receptor and cell kinetic analysis were submitted for further histological confirmation. Histological and cytological evaluation used to group tumors by variables relating to differentiation, proliferative activity, and multifactorial correlation with these assays will be the subject of a separate paper.

**Computed Analysis of Generated Data.** The data obtained from the tissue for steroid receptor results (molecular form and capacity), cell kinetic analyses (PI, % G1, % S, % G2), presence of aneuploid cells, and clinical data (age, lymph node status, and site of tumor) were obtained and stored on the General Electric DMS II data management system.

**RESULTS**

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Tissues was performed with objective computer programs and standardized definitions (see "Materials and Methods") which were designated prior to the analysis of the data. A correlation coefficient of 0.84 was obtained when comparing the % S activity with the PI activity (Chart 2A). A correlation coefficient of only 0.60 was obtained for the comparison of PI with the percent G2 fraction activity (Chart 2B). Because of the relatively high positive correlation between PI and % S, it was decided to use % S activity in most of the analysis as it is probably a more important biological parameter relating to cell kinetic activity, and since it would allow us to compare our data with similar studies using tritiated thymidine in breast cancer (20, 26, 27).

At the present time, we have not used G2 activity alone as described by Irvin and Bagwell (11) for cell kinetic analysis. In the 70 breast tumors analyzed for cell kinetics using flow cytometry, a wide distribution of % S activity was noted (Chart 3). There is a steady decrease in the distribution of patients as % S activity increases. The mean value of % S activity was 13.7 ± 6.5 (S.D.). In a series of 6 fibroadenomas obtained from Dr. Kenneth McCarty, Jr. (Duke University) and analyzed in our laboratory, the % S activity was 6.9 ± 1.8. None of these tumorous tissues, along with several normal breast tissues, showed any aneuploid activity. In the 70 breast cancers studied, aneuploidy was noted in 44%. The distribution of PI and % S values for diploid and aneuploid tumors is shown in Chart 4. Values for diploid tumors were 10.4 ± 5.5 for % S and 19.0 ± 7.1 for PI. Values for aneuploid tumors were 17.9 ± 5.1 for % S and 27.1 ± 6.2 for PI. Using t tests, the mean values for PI and % S are significantly different between the aneuploid and diploid groups to a p < 0.01 value. In 103 DNA histograms done on breast tumors in our laboratory, only one tumor has been proven to be hypodiploid as determined by mixing experiments. The average coefficient of variation for the G1 and G2 peaks in the DNA histogram was between 3 and 7% as determined from the Fried program. The DNA histogram for peripheral blood always gave a lower coefficient of variation for the G1 phase nuclei than did the DNA histograms for the tumor tissue.

Using a threshold level of 10 fmol/mg of protein as the value for a steroid receptor-positive tissue, overall analyses of the distribution of ER- and PR-positive tumors were 54 and 45%, respectively. Further distribution into ER-PR categories is shown in Table 1. The ER-positive-PR-positive group is approximately equal in number to the ER-negative-PR-negative group, while the ER-positive-PR-negative and ER-negative-PR-positive are significantly smaller in number. The distribution is in good agreement with previous investigations (17, 18) and would indicate that the 70 tumors analyzed are a good sampling of breast cancer tissue as related to steroid receptor analysis.

A comparison of the steroid receptor status with cell kinetics and % aneuploidy was performed with the data in Table 1 using an analysis of variance. There is evidence of a significant difference between the mean values of PI and % S between ER-positive-PR-positive and ER-negative-PR-negative tumors (p < 0.01). The mean values of % S for the ER-positive-PR-
negative group and ER-negative-PR-negative group are also significantly different (p < 0.01). However, there is no significant difference at the p < 0.05 level between the ER-negative-PR-positive and the ER-negative-PR-negative group with respect to PI or % S. The percentages aneuploid of ER-positive-PR-positive and ER-negative-PR-negative were not significantly different in occurrence as determined by $\chi^2$. However, the trend is for % S, PI, and % aneuploidy to increase as the tumors go from ER-positive-PR-positive to ER-negative-PR-positive to ER-negative-PR-negative. The range of % S for each subgroup of steroid receptor status is large. In this study, the ranges of % S values for ER-positive and ER-negative tumors are 2 to 23 and 4 to 27, respectively. The ranges of % S values for PR-positive and PR-negative tumors are 2 to 23 and 4 to 27, respectively.

Tumors were categorized as to ER positive, ER negative, PR positive, and PR negative and related to % S and % aneuploidy (Table 2). Again using $t$ tests, a statistically significant difference was noted in the % S mean value of ER-positive tumors (11.1 ± 5.2) versus ER-negative tumors (16.8 ± 6.4; p < 0.01). There was, however, no significant difference between the % S mean values of the PR-positive and PR-negative tumors.

Since 85% of the tumors were analyzed by sucre density gradients, the molecular forms of the steroid receptor could be obtained and compared with the cell kinetic data. The results of this comparison are summarized in Table 3. The mean % S value is lower in tumors which contain both 4S and 8S estrogen receptors than in tumors which have only the 4S estrogen receptor. If the tumor is negative for both 4S and 8S molecular forms, as defined by having less than 10 fmol of ER per mg of protein, the mean % S value is significantly higher. The mean % S value is lowest in tumors which have only the 8S molecular form of the PR. There is an increase in the mean % S value of tumors which have both forms of PR, but the highest % S is seen in patients without receptor. The tumors which have only the 4S PR have a mean % S value not significantly different from those tumors which contain neither the 8S nor 4S molecular forms of this steroid. The importance of steroid receptors and their molecular forms will be discussed later.

The relationship of cell kinetics and % aneuploidy was correlated with the clinical status of the patient (Table 4). Only patients who had 5 or more nodes examined at the time of primary treatment were included in this evaluation. Analysis of variance revealed no significant difference in kinetic parameters between the groups with respect to positive nodal status or metastatic disease. The number of positive nodes and metastatic disease was also compared with receptor status, and no statistically significant differences were observed. This is in agreement with other reported studies (24).
Other investigators have shown a relationship among more detailed cellular and nuclear characteristics, receptor activity, and cell kinetic data (3, 16). We are presently trying to confirm these data.

The distribution of % S activity for the 70 tumors (Chart 3) is very wide and does not seem to be in a lognormal distribution as described by other investigators (22). The % S activity for benign breast disease is significantly different when compared with breast cancer tissue. In the limited number of benign and normal tissues, none of the DNA histograms obtained showed aneuploid characteristics. However, the 70 breast cancer tissues studied here showed a 44% aneuploidy. This value is significantly lower than the 75 to 78% aneuploidy obtained by Barlogie's group (4, 25). The reason for this discrepancy is unknown at this time but could be due to methodology and equipment used, or sampling variability. A total of 103 tumors were analyzed in this study. Thirty-three of these tumors were insufficient for complete analysis of the parameters used in this study. Of these 33 tumors, 28 were aneuploid, giving a total percentage of aneuploids in this study of 57%. Of these 28 tumors, 7 had overlap of the diploid and aneuploid peaks, precluding histogram analysis. The remaining 70 patients form the basis of this report.

The relationship between PI and % S activity obtained from diploid and aneuploid tumors is significantly different (Chart 4). This would agree with the general hypothesis that more aneuploid tumors are usually more poorly differentiated histologically and usually faster growing. It is possible that a systematic error in the calculations could have been obtained from overlapping of the diploid histogram with the aneuploid histogram. At present, there are no computer programs available to correct for this possible error. The tumors analyzed which did not give a good separation between the diploid cells and the aneuploid cells were removed from any further analysis. It is also possible that the diploid histogram has a larger percentage of noncancerous cells which do not divide as rapidly. Finally, we plan to use vital DNA stains to sort out the different cell populations by DNA content using the capability of the fluorescence-activated cell sorter. This might answer our sampling problem and give us the potential to sort out monoclonal cells with aneuploid DNA content.

Our data support a firm relationship of steroid receptor status and cell kinetic parameters as defined by flow cytometry (Tables 1 and 2) and are in agreement with reports from other investigators (20, 25, 27). A greater PI, % S activity, and more frequent aneuploidy is associated with low steroid receptor activity and supports the hypothesis that steroid receptor activity is indirectly related to the proliferative capacity of breast cancer. Although the correlation between receptor activity and cell kinetic parameters is strong, considerable overlap has been noted. Further studies, will, hopefully, define whether PI or % S activity is related to response rates and duration in patients undergoing therapy. Protocols which use these data in a prospective manner are currently being developed at our institution.

Our data also indicate that the ER status is more predictive of lower % S activity than the PR status (Table 2). This is contrary to the general view that, since PR is a product of ER action, it should be more predictive of estrogen-sensitive cells. However, the present data by Knight et al. (13) and Meyer and Lee (22) indicate that ER-positive tumors are slow growing and that the patient usually has a better prognosis compared to the patients with the faster growing ER-negative tumors who have a poorer prognosis. Allegra et al. (1) have shown no relationship between prognosis of the patient and the presence of PR. This agrees with our data.

Patients whose tumors contain the 8S molecular form of the receptor have been shown to have a higher probability of response to hormone therapy (28). The relationship between the molecular forms of the steroid receptor and cell kinetics (Table 3) indicates that tumors which contain the 8S molecular species have a lower mean % S activity than do those which have only the 4S molecular form or neither molecular form. It should be noted, however, that the number of tumors in the molecular species categories is low and that there is wide distribution of % S activity in each category. Therefore, a relationship of cell kinetic activity and response to hormone therapy might exist through the use of molecular forms, but more analyses are needed.

A direct relationship of the actual capacity of steroid receptors (fmol/mg of protein) and the % S value was not observed. This has also been studied by Silvestrini et al. with similar results (27). Raber et al. (25) have found an inverse relationship between ER content and proliferative activity. There are several reasons why a direct relationship of this type may not exist. (a) An exact relationship between molecules of receptors per cell and biological activity has never been shown. If a threshold of receptors per cell is reached, it should be active as an estrogen-sensitive cell. Any more receptor molecules per cell would not enhance this biological activity. Therefore, the steroid receptor concentration would not be the limiting factor for steroid sensitivity or biological activity. (b) The assay of fmol/mg of protein is generally an inaccurate estimation of molecules of receptor per cell because of methodological considerations. These include dissociations of radioactive steroids from the receptor, nonsaturation of receptor, and use of mg of protein as a standardization procedure. It has been estimated that the mg of "cytosol" protein observed in a tumor extract can be contaminated with as much as 45% albumin obtained from blood and interstitial fluids (9). It is possible that cell kinetic data can be related to receptor content, but new methodologies must be developed.

The lack of correlation of cell kinetic data with age, nodal status, and metastatic disease is noteworthy. Our sample size was not large enough to detect small but possibly significant differences. In addition, the fact that this was a retrospective study may have biased our results in these analyses. Prospective studies which contain prognostic variables related to tumor size and more detailed histological characteristics will be necessary to adequately determine any relationship that might exist.

Flow cytometry as used in this study may prove useful as a tool in both the basic and clinical areas. It will be important to determine the role of cell kinetics in treatment planning and in the natural history of the disease and to determine whether kinetic data supply important independent clues that cannot be obtained by standard methods of evaluation.

ACKNOWLEDGMENTS

We acknowledge the members of the surgery and oncology staffs at Bowman Gray School of Medicine, especially Dr. Robert Cooper who initiated the cell
kinetic analysis work, and Dr. Leonard Rhyne who helped transfer the Fried program to our computer. We would also like to thank Dr. Ken McCarty, Jr., for supplying the benign breast tissues. Finally, we would like to thank Dr. Russell Hilf and Dr. Richard Marshall, who gave constructive comments on this manuscript.

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