Bioavailability of Estradiol as a Marker for Breast Cancer Risk Assessment¹

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ABSTRACT

The search for a hormonal marker in breast cancer has centered on estrogens and their metabolites. However, direct measurements of total amounts of these steroids have shown no convincing or consistent differences between normal women and women with breast cancer. The purpose of this study was to measure the percentages of non-protein-bound estradiol (%NPBE) and of estradiol bound to albumin (%ABE) and the levels of sex hormone-binding globulin (SHBG) both in women with breast cancer and in those free of disease. Serum was collected and analyzed within 2 weeks, using an isodialysis method. The mean %NPBE and %ABE were significantly higher in 32 women with breast cancer (1.73 and 64.0%, respectively) than in 32 matched disease-free women (1.43 and 48.6%, respectively) (P < 0.001). No significant difference was observed in the levels of plasma albumin when the above matched groups were compared. However, plasma levels of SHBG were significantly lower in the women with breast cancer than in either the control population or matched controls. In this finding we differ from previous studies which reported no significant differences in the mean plasma levels of SHBG. In our study, the increased %NPBE and %ABE in some patients with breast cancer may be related to a lower level of plasma SHBG; other factors, too, may affect the distribution of estradiol. Our results support the hypothesis than an increase in %NPBE and %ABE or both may indicate an increased risk of breast cancer.

INTRODUCTION

The evidence relating endocrine features to the etiology and pathogenesis of breast cancer is extensive (1–3). However, despite years of research, no specific difference in endocrine function has yet been identified between women who develop breast cancer and those who do not.

Only 25% of those women who develop breast cancer have been shown to have the known risk factors, i.e., obesity, family history, etc. Evidence is strong, however, that increased or prolonged estrogen stimulation is related to an increased risk of breast cancer (2). Researchers have also postulated that unopposed estrogen stimulation plays a principal role in increasing the risk of developing breast cancer (2). Some have suggested that this action of estrogen occurs predominantly during the periods of life when very small amounts of progesterone are present (3). These periods usually occur in the postmenarcheal years and in the pre- and perimenopausal years, times when luteal-deficient cycles are most likely to occur. However, no correlation between the two factors could be identified in geographic areas where both the incidence of breast cancer and luteal-deficient cycles were high (4).

Nevertheless, existing evidence implicates estrogen as playing an important role in the etiology and pathogenesis of breast cancer (5). It is important, therefore, to search for and evaluate the relevant differences between estrogen levels in women with and without breast cancer.

At present, no simple test or reliable biomarker is available for the identification of women who have or who are destined to develop breast cancer. Direct measurement of estrogens in sera and urine of patients and controls has failed to show consistent or convincing differences. Preliminary data reported by Osborne et al. (6) suggest that women with an increased risk of breast cancer have abnormally high rates of 16α-hydroxylase when compared to matched controls. Although this abnormality may well be a biomarker, this measurement requires hospitalization and injection of radio labeled substances. Recently, several investigators have used simple isodialysis techniques to demonstrate significant differences between the %NPBE in women with breast cancer and their matched controls (7–9). In addition, we recently reported an elevated %NPBE in 2 premenopausal women whose mammograms were negative but who subsequently developed breast cancer (10). Moore et al. (11) have reported similar findings in a larger group of women. In their prospective study, 18 disease-free women with a significantly elevated %NPBE subsequently developed breast cancer. The women in these studies were reported to be within a defined range of normal weight. Bruning et al. (12) reported findings which do not support the earlier studies. However, in a more recent report Bruning and Bonfrer (13) indicate that other factors such as diet, stress, or both may have contributed to the lack of a difference between their breast cancer patients and the controls.

In this study, the %NPBE and %ABE were measured in sera specifically collected for this study. Sera were obtained from patients with untreated breast cancer prior to surgery and from matched controls documented to be disease free by mammogram and physical examination. The results of the present study support our previous findings and those of others which demonstrated that the %NPBE is significantly elevated in women with breast cancer (7–11). The results presented here are a part of a larger ongoing epidemiological study to assess the bioavailability of estrogen in women with breast and gynecological cancers.

SUBJECTS AND METHODS

Patients and Normal Controls. Presurgical blood samples were obtained from 32 women (13 pre- and 19 postmenopausal) who had either stage I or stage II breast cancer at their initial visit to either the General Surgery Clinic at The University of Texas M. D. Anderson Hospital and Tumor Institute at Houston or the Kelsey-Seybold Clinic. Control sera were obtained from women who were seen in the breast cancer screening program at the Cancer Prevention Center of the Kelsey-Seybold Foundation. Both groups of women were initially given a lengthy epidemiological risk assessment questionnaire developed by the Department of Cancer Prevention at the M. D. Anderson facility. The questionnaire was reviewed with the patients by a nurse screener. The

¹ The abbreviations used are: %NPBE, percentage of non-protein-bound estradiol; %ABE, percentage of estradiol bound to albumin; SHBG, sex hormone-binding globulin.

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women selected as a general control population underwent mammography in accordance with the American Cancer Society guidelines. Controls selected for inclusion in the screening study were disease free (i.e., their mammograms were negative for breast disease), had no endocrine disorder, and had not been taking any hormonal medication or drug known to affect plasma levels of SHBG within the 6 months preceding collection of blood. Obesity alone was not a criterion for exclusion.

One hundred eighty-eight women comprised the total control group. Of these, 32 were matched individually to the 32 patients on the basis of age (±3 years); menopausal status; body weight and height; age at first pregnancy (before or after age 30); parity (nulliparous, 0 or 2 children, and 3 or more children); socioeconomic status, based on years of education; and family history of breast cancer. Menopausal status was determined by responses to questions in the questionnaire and on total estrogen and progesterone serum levels. A woman was considered postmenopausal if she met one of the following criteria: (a) over 45 years old with no menses for 6 months prior to inclusion into the study and an estradiol plasma level of less than 40 pg/ml; or (b) surgical menopause. Stage of the cycle was based on the date of the last menses and the plasma level of progesterone. Additionally, although previous studies by Hammond et al. (14) and by Jones and Bauman have shown that %NPBE is not affected by time of day, the stage of the menstrual cycle, or menopausal status, premenopausal patients were matched with controls by the stage of their cycle.

The blood samples were obtained between 8 a.m. and 4 p.m. They were allowed to clot at 4°C for 24 h and were then centrifuged. The sera were removed, aliquoted, and stored at −20°C until assayed. The maximum length of storage prior to assay was 2 weeks. Additional serum samples were stored at −70°C. According to data published by Langley et al. (15), 2 weeks are well within the maximum time allowable for determining %NPBE and %ABE before samples degrade. Ideal body weight was calculated according to the following formula based on the Metropolitan Life Insurance Company weight tables (16):

\[
\text{Ideal body wt} = 105 \text{ lb} + 5 \text{ lb for each inch over 60 inches}
\]

Analytical Methods. The sera were analyzed using a previously described isodialysis method (14). In brief, 450-μl aliquots were incubated for 1 h at 37°C with [3H]estradiol purified by column chromatography within 24 h of the assay and [14C]glucose. Duplicate aliquots (200 μl) of the serum were placed in modified 12- × 75-mm baked test tubes, which were closed at one end with a specially treated dialysis membrane and open at the other end. The modified test tubes were then placed in baked minivials. Each minivial contained 3 Whatman No. 1 filter disks at the bottom. The minivials were baked and then centrifuged at 37°C for 1 h at 3000 rpm in a Beckman J6B low-speed centrifuge. After centrifugation, the inner tubes were removed and 30 μl of serum were removed and placed in a separate minivial. Double-distilled water (350 μl) was added to each minivial and the minivials were vortexed. After 15 min, scintillation fluid was added and the contents of the vials were then counted in a refrigerated Beckman LS 7500 scintillation counter.

The %NPBE was determined as

\[
\%\text{NPBE} = \frac{3\text{H:14C}_{(\text{outside})}}{3\text{H:14C}_{(\text{inside})}} \times 100
\]

The %ABE can be determined from the comparison of the %NPBE measured in native and heat-treated serum (17). A 90% loss in the binding capacity of SHBG results from heating serum to 60°C for 1 h while the binding of estradiol to pure human serum-albumin solutions is unaffected (18). It is possible to determine the relative amount (percentage) of steroid that interacts with heat-stable binding proteins in the absence of high-affinity binding by SHBG, by measuring the %NPBE in heat-treated serum samples.

The levels of plasma SHBG were determined by a liquid-phase immunoradiometric assay using an anti-human SHBG monoclonal antibody provided by Dr. Geoffrey Hammond, Department of Obstetrics and Gynecology, University of Western Ontario, Canada (18).

Serum estrogen and progesterone levels were determined by radioimmunoassay kits (estradiol, Pantex, Santa Monica, CA; progesterone, Cambridge, Boston, MA). Plasma albumin levels were determined using the method of Rodkey (19).

All assays included at least 2 sets of control sera. The mean intraassay coefficient of variation (of duplicate determinations) for estimating the %NPBE over a range of 36 high-to-low values was 10.0%. Comparison of the %NPBE and %ABE measurements of control sera stored longer than 1 year showed coefficients of variation of less than 12 and 8%, respectively (n = 12). Blood samples were drawn randomly from 8 premenopausal control women over a period of 6 months. A comparison of the %NPBE and %ABE values obtained from individual women over this period of time had coefficients of variation of less than 6.3 and 5.2%, respectively. Therefore, single sample determinations are reported here. Statistical analysis involved a 2-tailed Student’s t test (paired and unpaired where applicable).

RESULTS

Mean (±SE) serum %NPBE, %ABE, and concentrations of SHBG, and albumin along with the age and deviation from ideal body weight for women with breast cancer and matched control subjects are presented in Table 1. The %NPBE values of 1.50 ± 0.02 and %ABE values of 49.6 ± 1.9 were observed in the 188 women who comprised the general control population. In addition, the %NPBE and the %ABE in the general control population were correlated with weight but not with age or menopausal status. The mean (±SE) deviation from ideal body weight of normal-weight breast cancer patients and matched controls was 1.9 ± 2.9 and 1.7 ± 3.3, respectively. Obese cancer patients and the matched controls exhibited a mean (±SE) deviation from ideal body weight of 62.0 ± 9.9 versus 54.1 ± 6.4, respectively.

As shown in Table 1, the difference between the %NPBE in breast cancer patients and the matched controls (1.73 ± 0.05 versus 1.43 ± 0.05) was highly significant (P < 0.001). When menopausal status was considered, the %NPBE of both the 13 pre- and the 19 postmenopausal patients differed significantly from that of the matched controls (P < 0.02 and P < 0.01, respectively) (Table 2). However, 12 of the 19 postmenopausal patients were more than 20 pounds over ideal body weight. Even when the comparisons were made by weight, the %NPBE of obese women with breast cancer was significantly different (P < 0.05) from matched controls for both the 3 pre- and 12 postmenopausal women as shown in Table 3. An even larger difference was seen when the comparison was restricted to normal weight women (within approximately 20 pounds of ideal body weight). A %NPBE value of 1.72 ± 0.07 in 17 normal weight cancer patients was significantly higher than the 1.31 ± 0.005 observed in matched normal weight controls (P < 0.001) (Table 3). Although no significant difference was evident between the %NPBE of normal weight and obese patients with breast cancer (1.72 ± 0.07 versus 1.74 ± 0.05), the %NPBE of the respective matched control group was significantly different (1.31 ± 0.005 versus 1.55 ± 0.5; P < 0.05).

The %ABE in women with breast cancer and the matched controls is summarized in Table 1. The mean %ABE of the 32 breast cancer patients was 64.0 ± 2.0 compared with 48.6 ± 2.3 for the matched controls (P < 0.001). There was no difference between the %ABE in the pre- and postmenopausal cancer patients. A significant elevation in the %ABE was found in the 15 obese patients when compared to that of the obese matched controls (P < 0.05) (Table 3). Similar differences were observed in the 17 normal-weight patients and their matched controls (P < 0.001) (Table 3).
A highly significant correlation \((r = 0.71, P < 0.001)\) between the \%NPBE and the \%ABE in 188 general controls is shown in Fig. 1. This correlation was observed for both the women with breast cancer \((r = 0.56, P < 0.001)\) as well as the matched controls \((r = 0.82, P < 0.001)\) (Fig. 2). There was no significant difference between the slope of regression lines derived from the patients and their matched controls. Interestingly, there was no correlation between the deviation from ideal body weight and either \%NPBE or \%ABE (Figs. 3 and 4) in the breast cancer patients. However, a significant correlation between both \%NPBE and \%ABE and deviation from ideal body weight was found in the matched controls (Figs. 3 and 4).
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Fig. 1. Relationship between percentage of non-protein-bound estradiol and percentage of albumin-bound estradiol in 188 disease-free women. The correlation coefficient \( r \) is highly significant \( (r = 0.71, P < 0.001) \).

Fig. 2. Relationship of percentage of albumin-bound estradiol to percentage of non-protein-bound estradiol in matched controls \( (\bullet) \) and breast cancer patients \( (\odot) \). The correlation coefficients \( r \) are highly significant \( (\text{matched controls, } r = 0.82 \ (\text{Line A}), P < 0.001; \text{breast cancer, } r = 0.56 \ (\text{Line B}), P < 0.001) \).

Fig. 3. Relationship of ideal body weight to percentage of non-protein-bound estradiol in matched controls \( (\bullet) \) and breast cancer patients \( (\odot) \). Ideal body weight is calculated by subtracting the difference of actual body weight from calculated ideal body weight. The correlation coefficient \( r \) of the matched controls is significant \( (r = 0.45 \ (\text{Line A}), P < 0.005) \), while that of the breast cancer patients is not \( (r = 0.21 \ (\text{Line B})) \).

Fig. 4. Relationship of ideal body weight to percentage of albumin-bound estradiol in matched controls \( (\bullet) \) and breast cancer patients \( (\odot) \). Ideal body weight is calculated by subtracting the difference of actual body weight from calculated ideal body weight. The correlation coefficient \( r \) of the matched controls in significant \( (r = 0.49 \ (\text{Line A}), P < 0.005) \), while that of the breast cancer patients is not \( (r = 0.24 \ (\text{Line B})) \).

The influence of plasma SHBG concentrations on the \%ABE of the patients and matched controls was found to have a significant inverse correlation for both groups \( (r = -0.69, P < 0.001; r = -0.65, P < 0.001) \) (data not shown). No significant difference was found when the slopes of these regression lines were compared.

DISCUSSION

A number of investigations have documented significantly elevated levels of unbound estradiol in persons with breast cancer \( (7-9) \). The validity of some of these results has been questioned because of the length of sample storage prior to analysis and the uncertainty about whether the samples were obtained before or after surgery. In addition, a recent study by Bruning et al. \( (12) \) found no significant difference in the \%NPBE when comparing the results from assays of serum samples from postmenopausal women with and without breast cancer and from postsurgical samples from premenopausal women with breast cancer and those from normal controls. Our results using fresh sera from both patients with breast cancer and matched disease-free controls lend support to the previous findings that elevated levels of \%NPBE are present in patients with breast cancer.

Measurement of the non-protein-bound steroid concentrations in sera is now widespread. These measurements are based on the premise that the non-protein-bound fraction is biologically active and that this fraction may readily correlate with clinical observations. Although few such correlations have been found, a number of studies, including our own, have shown a strong correlation between an elevated NPBE fraction and the occurrence of breast cancer \( (10, 11) \). In addition, our group \( (10) \) as well as that of Moore et al. \( (11) \) have shown that these elevated levels may occur prior to the diagnosis of breast cancer. Our results also indicate that the elevation of \%NPBE may be related to a decrease in SHBG plasma levels in some of these patients. Although this paper presents no current data about other factors such as high-lipid-containing diets, evidence indicates that, in both the presence and absence of obesity, such factors may also lead to increased \%NPBE. The present data do show a positive correlation between an increased \%NPBE and elevated \%ABE and therefore suggest that \%ABE may play an even more important role than \%NPBE in the bioavailability of estrogen in the etiology of breast cancer.

Although similar reports of an elevation in the \%NPBE and the \%ABE have appeared in the literature \( (7-11) \), the controversy continues as to what may account for the differences. One possible explanation may be the method of sera collection. The sera utilized in most studies have been collected for purposes other than the study. These sera may have been stored at \(-20^\circ C\) or diluted prior to storage or analysis. Both of these factors have been shown to affect the binding kinetics of specific serum proteins \( (12, 14) \). Differences may also result from variability in assay conditions. An elevation in temperature has been shown to have an effect on the \%NPBE \( (14) \). Centrifugation of
samples at temperatures above 37°C may result in an elevation of %NPBE values above those of the same samples centrifuged at 37°C.\(^4\) We have also shown that as the length of time between purification of \(^{1}H\)estradiol and analysis increases, so also do %NPBE values. In a series of experiments measuring sera with either high or low %NPBE values, the longer the time span between purification of the \(^{1}H\)estradiol and analysis, the closer the lower and higher values approximated one another.\(^5\) It is crucial, therefore, that the proper temperature be maintained and that the \(^{1}H\)estradiol in the analysis be purified within 24 h of use.

Unlike previous studies, both obese and normal-weight breast cancer patients were included in our study insasmuch as a number of reports have shown that obesity and an elevated %NPBE are directly related (21–23). Nisker et al. (21) reported that the %NPBE was significantly elevated and the plasma level of SHBG was significantly lower in obese patients with endometrial cancer than in normal-weight controls. No significant difference was observed in either the %NPBE or the plasma SHBG level when these values were compared in the obese group of cancer patients to those of the matched obese controls. In the current study, the mean %NPBE of the obese women with breast cancer was significantly greater than that of the matched controls. More important, a significantly greater mean %ABE in both obese and normal-weight women with breast cancer was observed. The %ABE in breast cancer patients was significantly greater than that of either the general controls or the respective matched controls. Moreover, the mean %ABE in normal-weight and obese controls were significantly different, while these same parameters in the patient groups were not. The %NPBE and %ABE of normal-weight postmenopausal patients and of their matched controls are similar to those previously published (14).

Recent studies by Partridge (23) and Manni et al. (24) suggest that the biologically active fraction of such steroids as estradiol and testosterone includes both the non-protein-bound and albumin-bound fractions. On the basis of their clinical studies, Cumming and Wall (25) have recently suggested that the non-SHBG-bound testosterone may be the optimal marker for androgen excess in women. It is our belief that this may also be true for non-SHBG-bound estradiol in breast cancer.

Recently, Moore et al. (26) compared the distribution of estradiol between that bound to SHBG and albumin in normal disease-free British women from the island of Guernsey and normal disease-free semirural Japanese women. The Japanese women had more estradiol bound to SHBG than did the British women, although the mean %NPBE was the same. Moore et al. (26) proposed that this shift to albumin may be either genetic or dietary. However, their study demonstrated clear difference between two populations which differ in the incidence of breast cancer. As suggested by Moore et al. (26), this could be an explanation for the increased risk of breast cancer among British women. Our control population of normal American women had a %ABE similar to that recently reported by Langley et al. (15) for a group of disease-free British women. Nunez et al. (27) has suggested that the nonesterified fatty acid-binding properties of albumin may modulate estrogen action during normal or neoplastic development. Consumption of dietary fats has been linked to the greater incidence of breast cancer found in Western women. Reed et al. (28) have shown that unsaturated free fatty acids can increase the bioavailable estradiol fraction in plasma in vitro. A recent in vivo study by Brunning and Bonfner (13) strongly supports the hypothesis that plasma free fatty acids, which normally bind to albumin, can influence plasma non-protein-bound estradiol. This study also points to the effect of diet, feeding states, and possibly stress on non-protein-bound estradiol concentrations and may also explain the discrepancies between previously reported studies, our present data, and those of Bruning et al. (12).

In summary, our findings support the hypothesis that an alteration in the distribution of estradiol may influence the bioavailability of this steroid. In particular, both the fraction which is non-protein-bound and that which is bound to albumin may be important in the etiology of breast cancer. Additionally, these results point to the potential usefulness of estradiol measurements, especially the measurement of %ABE, as biomarkers for the risk of breast cancer development.

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