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Estramustine Binding Site in Human Breast Cancer Biopsy Samples. Its Relation to Estrogen and Progesterone Receptor Levels, Age and Menopausal Status

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Abstract—*Estramustine is a cytotoxic metabolite of estramustine phosphate (Estracyt®), which is used in the treatment of prostatic carcinoma. An estramustine binding site (EMBS) at pH 4.8–4.9 was demonstrated in 74 of 306 (24%) breast cancer biopsy samples using isoelectric focusing in polyacrylamide gels. The presence of EMBS was significantly ($P < 0.001$) correlated with negative or low estrogen and progesterone receptor values. EMBS positivity was found in 31% of the samples from pre- and perimenopausal patients and in 22% of the samples from postmenopausals. If patients were instead divided into different age groups, EMBS positivity was most frequent in samples from patients between 50 and 59 years of age (42%). With increasing age the percentage of EMBS positivity fell successively. For patients under 50 years, no difference with respect to EMBS positivity between age groups could be demonstrated. The possible value of EMBS determinations in breast cancer tissue specimens for the selection of those patients that will respond to Estracyt® therapy should be evaluated.*

INTRODUCTION

ESTRAMUSTINE PHOSPHATE (EMP, Estracyt®) is a nornitrogen mustard derivative of estradiol-17β-phosphate, which has been used since 1966 for the treatment of prostatic carcinoma [1]. In the body EMP is rapidly dephosphorylated to estramustine, which is oxidized to a higher extent to the estrone counterpart of estramustine, estromustine [2]. Estramustine and most probably estromustine are believed to exert cytotoxic activity through an interaction with microtubule-associated proteins, inducing mitotic arrest of cells in metaphase [3, 4] and by interaction with the nuclear protein matrix causing cell death at the level of DNA replication [5].

An explanation for the accumulation of cytotoxic EMP metabolites in prostatic tumor cells was found with the detection of a protein, binding estramustine and estromustine specifically and with high affinity [6, 7]. This protein was called 'estramustine-binding protein' (EMBP) due to the unique binding affinity for these ligands. Though the biological function of this protein is not fully understood, proteins in the rat ventral prostate that most probably are identical to EMBP have also been described by others: 'α-protein' [8]; 'prostatic binding protein' [9]; 'prostatein' [10]; 'prostatic secretion protein' [11].

A few clinical trials with EMP in the therapy of other malignancies (e.g. malignant melanoma, renal cell carcinoma and breast cancer) have also been reported [12–16]. In the case of breast cancer, three investigations have shown response rates in 17/44, 2/16 and 2/34, respectively, of patients with advanced disease [14–16].

In order to find a prerequisite for EMP treatment of breast cancer and to explain why only certain patients respond to EMP therapy, the binding of estramustine in breast cancer biopsy samples has been investigated in order to find a binding site, similar to EMBP in the prostatic tissue. This study has been performed in parallel to the routine measurement of estrogen and progesterone receptors.

MATERIALS AND METHODS

Tissue material

Estramustine binding site (EMBS) and estrogen receptor (ER) have been examined in 306 and progesterone receptor (PgR) in 294 consecutive

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breast cancer biopsy samples. Eighty-three of the patients were classified as pre- or perimenopausal (less than 5 years after the last regular menstrual bleeding) and 223 as postmenopausal (more than 5 years after the last regular menstrual bleeding). Biopsy material was obtained at operation and was freed from fat, blood clots etc., minced and then kept for not more than 2 weeks at either −70°C or in liquid nitrogen until analysis.

Chemicals and ligands

All chemicals were of analytical grade. [2,4,6,7-^{3}H]Estradiol (91–93 Ci/mmol) was obtained from Amersham International, Buckinghamshire, U.K. [^{3}H]Promegestone ([^{3}H]R 5020, 87 Ci/mmol) and non-radioactive promegestone were purchased from New England Nuclear, Boston, MA, U.S.A. Non-radioactive estradiol and estramustine, and [2,4,6,7-^{3}H]estramustine (104 Ci/mmol) were kindly supplied by AB LEO Research Laboratories, Helsingborg, Sweden.

Analytical procedures

Tumor tissue was homogenized with an all-glass Potter–Elvehjem homogenizer in TEM-buffer (Tris–HCl 10 mM, EDTA 1.5 mM, mercaptoethanol 10 mM, pH 7.4) at 0°C. Centrifugation of the homogenate was carried out at 20,000 ***g*** for 10 min. The concentration of proteins was adjusted to 1–3 mg/ml and aliquots were analyzed for ER, PgR and EMBS as described below.

ER was measured with isoelectric focusing (IF) in polyacrylamide gels and PgR with the multiple point dextran-coated charcoal (DCC) method and Scatchard analysis as described previously [17].

Analysis of EMBS was performed with the IF technique according to the following brief description: [^{3}H]estramustine dissolved in absolute ethanol was added to the 20,000 ***g*** supernatant to a final concentration of 7.5 nM (final ethanol concentration was 3.2%). After 2 h incubation at 0°C, the sample was treated with DCC [dextran : charcoal 0.03 : 0.3 (mg/ml), final concentrations] for 1 min followed by a centrifugation at 3000 ***g*** for 10 min. The supernatant was applied in polyacrylamide gels and run as described previously [17]. The radioactivity was measured over the pH range 3.5–9.5.

Human serum albumin (HSA) has been reported to inferere with the binding assay for estramustine in human prostatic samples [7]. Therefore, for control, HSA was labelled with [^{3}H]estramustine and analyzed by the IF technique above.

The concentrations of ER and PgR were expressed as femto(f)mol specifically bound [^{3}H]estradiol (ER) and [^{3}H]R 5020 (PgR) per mg protein. Concentrations below 10 fmol ER/mg protein and 30 fmol PgR/mg protein were considered negative or low (ER− and PgR−). The amount of EMBS was only stated as positive or negative (EMBS+ or EMBS−). The protein concentration was measured according to the method described by Lowry *et al.* [18].

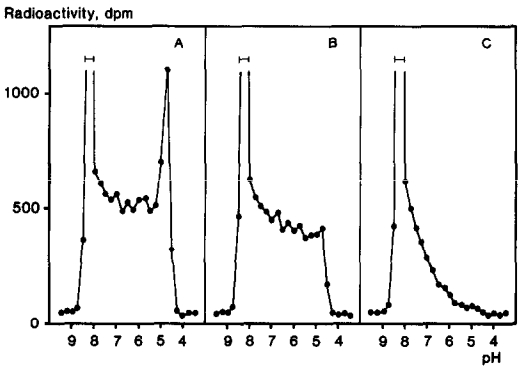

Fig. 1. Representative chromatograms from EMBS assays with isoelectric focusing in polyacrylamide gels.

Statistics

Evaluation of differences was made with chi-square analysis and *P*-values above 0.05 were considered as not having statistical significance.

RESULTS

Representative chromatograms from EMBS assays are shown in Fig. 1a–c. Figure 1a demonstrates, besides the application peak at pH 8, a distinct peak of [^{3}H]estramustine radioactivity at pH 4.8–4.9, preceded by a plateau exceeding background activity, probably consisting of ^{3}H-ligand dissociated from its binding site at non-optimum pH. In Fig. 1c significant radioactivity at pH 4.8–4.9 is absent. The chromatogram in Fig. 1b shows an intermediate appearance with no obvious peak at the appropriate pH, but a plateau of radioactivity clearly distinguished from the background activity as seen in Fig. 1c. In control experiments it was shown that samples with a distinct radioactive peak as in Fig. 1a gave the same type of chromatograms as shown in Fig. 1b when diluted with an EMBS− sample and with no major change in protein concentration (data not shown, four experiments). Due to these observations, samples with radioactive profiles as in Fig. 1a (n = 50) and 1b (n = 24) are referred to as EMBS+, whereas samples with the appearance as in Fig. 1c (n = 232) consequently are classified as EMBS−.

HSA labelled with [^{3}H]estramustine was focused around pH 5.2, and therefore no interference with the qualitative evalution of the EMBS assay was obtained (data not shown).

In the present investigation 74/306 (24%) breast cancer samples were EMBS+ according to the

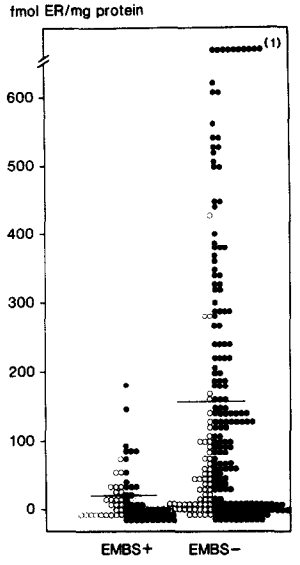

Fig. 2. Distribution of ER concentrations (fmol/mg *protein*) *in EMBS+ and EMBS− samples. Mean values are marked with arrows in the figure. Samples from pre- and perimenopausal patients are marked with* ○, *whereas samples from postmenopausal patients are marked with* ●. (1) 700–1300 fmol/mg *protein.*

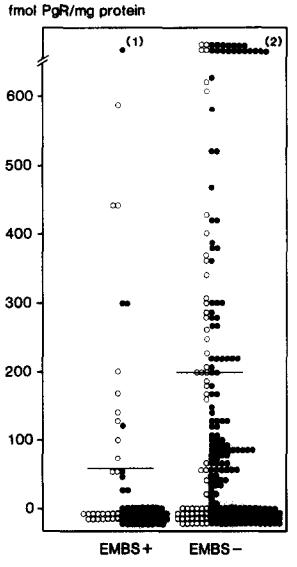

Fig. 3. Distribution of PgR concentrations (fmol/mg *protein*) *in EBMS+ and EMBS− samples. Mean values are marked with arrows in the figure. Samples from pre- and perimenopausal patients are marked with* ○, *whereas samples from postmenopausal patients are marked with* ●. (1) 850 fmol/mg *protein*; (2) 660–2300 fmol/mg *protein.*

definitions above. In Figs 2 and 3 ER and PgR concentrations are plotted for EMBS+ and EMBS− samples. Figure 2 shows that EMBS+ samples have lower mean ± S.D. ER values (20 ± 35 fmol ER/mg protein) compared with EMBS− samples (160 ± 220 fmol ER/mg protein). The corresponding figures for PgR were 57 ± 150 and 200 ± 340, respectively (Fig. 3). Figures 2 and 3 also show that EMBS+ was overrepresented in ER− and PgR− samples. Thus, in this study, 47/119 (39%) ER− samples contained EMBS,

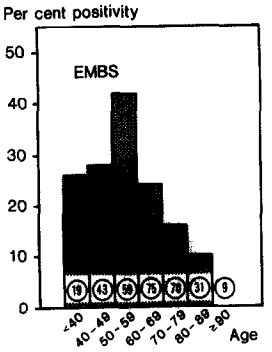

Fig. 4. Percentage EMBS positive breast cancer samples in relation to patient age at operation. The number of samples in each age group is indicated in the bars.

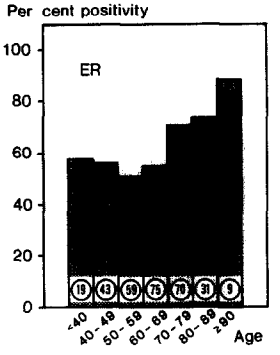

Fig. 5. Percentage ER positive breast cancer samples in relation to patient age at operation. The number of samples in each age group is indicated in the bars.

whereas only 27/187 (14%) ER positive (ER+) samples were EMBS+ ($P < 0.001$). The corresponding figures concerning PgR were 55/147 (37%; PgR− samples) and 17/147 (12%; PgR+ samples), respectively ($P < 0.001$). The proportion of EMBS+ in samples from pre- and perimenopausal patients was 26/83 (31%), whereas 48/223 (22%) samples from postmenopausal patients were EMBS+.

Figure 4 shows the distribution of EMBS+ samples with age. The highest frequency of EMBS+ (42%) was found in samples from patients between 50 and 59 years of age. As this group is heterogeneous with regard to menopausal status, the frequency of EMBS+ in this group was studied for both pre/peri- and postmenopausal patients. Ten of 22 (45%) of the tumor samples from pre- and perimenopausal patients were then found to be EMBS+. The corresponding figures for postmenopausals were 15/37 (41%). After the EMBS+ peak between 50 and 59 years the frequency fell with increasing age and for samples from patients 80 years of age or older only 3/40 (7.5%) were EMBS+. For patients under 50 years, no difference with respect to EMBS positivity between age groups could be demonstrated.

Figures 5 and 6 show the age distribution patterns

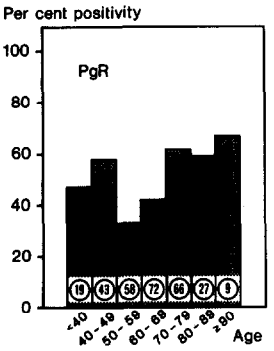

Fig. 6. Percentage PgR positive breast cancer samples in relation to patient age at operation. The number of samples in each age group is indicated in the bars.

Table 1. Correlations between EMBS and ER status in breast cancer tumor samples from pre/peri- and postmenopausal patients. (The figures in the table represent the number of samples)

	Pre/perimenopausal		Postmenopausal	
	EMBS+	EMBS−	EMBS+	EMBS−
ER+	12	35	15	125
ER−	14	22	33	50
	$P = 0.19$ NS		$P < 0.001$	

Table 2. Correlations between EMBS and PgR status in breast cancer tumor samples from pre/peri- and postmenopausal patients. (The figures in the table represent the number of samples)

	Pre/perimenopausal		Postmenopausal	
	EMBS+	EMBS−	EMBS+	EMBS−
PgR+	11	30	6	100
PgR−	15	27	40	65
	$P = 0.38$ NS		$P < 0.001$	

for ER+ and PgR+ samples, respectively. For ER+ and PgR+ samples, qualitatively different age distribution profiles as compared to EMBS+ samples were obtained. While a peak value for EMBS+ was demonstrated between 50 and 59 years, the frequencies for ER+ and PgR+ were the lowest in this group (51% and 33%, respectively). For patients older than 59 years an increase in receptor positivity with increasing age was obtained.

When examining the distribution of EMBS both in relation to menopausal and receptor status, a statistically significant correlation between EMBS+ and low or negative receptor concentrations was found only for postmenopausal patients (Tables 1 and 2).

DISCUSSION

The present investigation has demonstrated an estramustine binding site (EMBS) in 74/306 (24%) consecutive breast cancer biopsy samples. EMBS was characterized as a radioactive peak at pH 4.8–4.9 using isoelectric focusing in polyacrylamide gels. A similar p*I* (4.7–4.8) with the same technique has been reported for EMBP in rat and human prostatic tissue [7]. EMBS+ was found to be strongly correlated with ER− and PgR−. When studying the frequency of EMBS+ in relation to the patient age, EMBS+ was most frequent in samples from patients between 50 and 59 years of age. In this age group no obvious difference in the frequency of EMBS+ was found between pre/peri- and postmenopausal patients. However, since in this study the perimenopausal period has been defined as up to 5 years after the last regular menstrual bleeding, one can assume that patients from the age group between 50 and 59 years in general should have a hormonal status representative for menopausal or postmenopausal women. Samples from older postmenopausal patients showed a successively decreasing percentage of EMBS+ and it is worth mentioning that none of the 29 tumor samples from patients older than 82 years was EMBS+. With regard to ER and PgR status the opposite pattern was obtained. Samples from older postmenopausal patients were more often receptor positive than samples from younger postmenopausals. When EMBS status was correlated with receptor status, a statistical significant correlation between EMBS+ and low or negative receptor values could only be established for postmenopausal patients.

It is possible that the observed differences in EMBS and receptor status may reflect the changes in the hormonal milieu which appear during and after the menopause. The menopausal ovarian function is characterized by a fall in estrogen and progesterone secretion [19]. Furthermore, it has been suggested that the primary function of the postmenopausal ovary is to secrete androgens and androgen precursors, which should contribute to a shift to a more androgenic milieu after the menopause [20]. Such hormonal changes may be of relevance for the EMBS+ peak observed in the present work, as well as the low frequency of receptor positivity in samples from patients between 50 and 59 years. In this context it is also interesting that, in the ventral prostate from rat, EMBP was found to be dependent on androgenic stimuli [21, 22]. The concentration of EMBP showed marked changes as a function of age, ranging from very low levels in immature rats to a more than 200-fold increased levels in adults. After castration the concentration of EMBP dropped to less than 10% of precastration levels, but returned to normal levels after 2 weeks of androgen treatment [21]. It would be of interest to investigate the possible role of androgens for EMBS synthesis in breast cancer. An indication of

an immunological similarity between EMBS and EMBP have been observed in preliminary experiments, as both polyclonal and monoclonal antibodies against rat EMBP in some cases recognized an antigen in breast cancer cytosols [23]. Further work is necessary to show this similarity.

In this work no attempts have been made to quantitate the relative amount of EMBS. IF may not be the ideal method for this purpose as the radioactive peak appears at a non-optimum pH for the binding of [^{3}H]estramustine and is preceded by a plateau consisting of estramustine dissociating during the isoelectric focusing. The optimal pH for [^{3}H]estramustine binding in ventral rat prostate is 7.85 [24]. However, as indicated in the Results, different concentrations of EMBS resulted in differences in the chromatograms (Fig. 1a and 1b). Furthermore, variations in EMBS concentration have also been indicated when studying the height of the radioactive peak in the samples with an appearance as in Fig. 1a. In this context it should also be mentioned that preliminary experiments in our laboratory have indicated that the presence of endogenous ligand(s) may be of importance for the quantification of EMBS. Treatment with dextran-coated charcoal (DCC), prior to incubation with [^{3}H]estramustine have in some cases resulted in a higher degree of binding activity (data not shown). The presence of the endogenous ligand(s) may also be the reason why excess non-radioactive estramustine did not significiantly reduce the radioactive peak at pH 4.8–4.9. An alternative method to IF for EMBS measurement seems to be ion exchange fast protein liquid chromatography (FPLC; Pharmacia, Uppsala, Sweden). With this technique the specificity of EMBS has been studied. A 1000-fold excess of non-radioactive estramustine has in 10 samples, pretreated with DCC, to a varying degree (0–85%) displaced the radioactive peaks eluted with 0.10–0.40 M KCl (data not shown).

It is now well established that there is a positive correlation between receptor content and the effect of endocrine therapy in patients with disseminated breast cancer [25]. Of patients with ER and PgR positive tumor samples, about 70% will respond to hormonal treatment, whereas less than 10% without significant amounts of receptors will respond to this therapy [26]. Consequently, the latter group will usually be given cytotoxic drugs as first line therapy. For this group, however, it seems as if the study reported here should motivate a clinical investigation on the effects of estramustine phosphate in postmenopausal breast cancer patients with ER− and PgR− tumors. In this study EMBS would be measured in order to evaluate its significance for the clinical efficacy of Estracyt® in breast cancer treatment. Such a study has now started in the Southern Swedish Health Care Region.

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