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Analysis of Cytosolic Phosphoethanolamine and Ethanolamine and Their Correlation with Prognostic Factors in Breast Cancer

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Availability of accurate prognostic factors is vital in making decisions on cancer therapy. We have measured the cytosolic contents of phosphoethanolamine and ethanolamine in tumor tissues of 53 breast cancer patients in an attempt to explore the possibility that these amines could be used as prognostic indicators. The levels of phosphoethanolamine and ethanolamine were determined by high-performance liquid chromatography. The ratios of the molar quantity of these amines or amino acids to that of alanine plus tyrosine, which eluted as a single peak, were used to analyze and compare the results among different tumor samples. The results indicated that the values for phosphoethanolamine or ethanolamine varied significantly more than the values for amino acids, such as glycine plus threonine or glutamine plus serine (these amino acids were eluted as single peaks, respectively). The values for phosphoethanolamine, ethanolamine, and phosphoethanolamine plus ethanolamine were analyzed in relation to several commonly used prognostic factors of breast disease. The results indicated that groups having higher mitotic indices had significantly higher values for phosphoethanolamine or phosphoethanolamine plus ethanolamine than the group having lower mitotic indices. As the stage of the disease increased, the values for phosphoethanolamine plus ethanolamine also seemed to become higher. No correlation, however, was observed between steroid hormone receptor positive and negative groups or between positive and negative groups with regard to involved axillary lymph nodes. The content of phosphoethanolamine or phosphoethanolamine plus ethanolamine in cytosol therefore seems to be correlated with some prognostic indicators.

Key words: Phosphoethanolamine — Ethanolamine — Breast cancer

It is essential to have reliable markers that can predict the course of disease at the time of diagnosis in order to successfully treat cancer. Tumor grading, steroid receptor status, flow cytometry analysis of ploidy, mitotic index, oncogene or cathepsin D status, etc., have been proven to be significant prognostic factors of breast cancer.[1–5] Since tumors are variable in their properties, it is desirable to have as many prognostic markers as possible to determine the strategy of treatment.

Normal mammary epithelial cells require ethanolamine (Etn) to proliferate optimally in culture. Some neoplastic mammary epithelial cells also require Etn to grow (Etn-responsive), though other neoplastic mammary epithelial cells grow well without Etn (Etn-nonresponsive).[6,7] Etn-responsive mammary carcinoma cell lines such as T47-D human breast carcinoma and 64-24 rat mammary carcinoma cells tend to be of less progressive type (steroid receptor-positive and prolactin- and steroid hormone-responsive) as compared to Etn-nonresponsive cell lines such as MDA-MB-231 human breast carcinoma and 22-1 rat mammary carcinoma cells.[6,7]

The composition of cellular membrane phospholipid of Etn-requiring cells is similar to that of *in vivo* tissue when they are grown in medium supplemented with Etn.[8] However, in the absence of supplemental Etn, the content of phosphatidylethanolamine (PE) in their membrane becomes 1/2 to 1/3 of the normal amount and that of phosphatidylcholine (PC) increases by 30%.[8,9] The rate of growth slows down as the PE content is reduced and growth eventually stops.[8] The aqueous pools of Etn and phosphoethanolamine (PEtn) have been compared in Etn-responsive and -nonresponsive cells in culture, showing that Etn-nonresponsive cells have pools ten-fold larger than those of Etn-responsive cells at various concentrations of supplemented Etn.[8]

Based on the observations that (1) mammary carcinoma cells having characteristics of progressed malignancy tend to be Etn-nonresponsive and that (2) Etn-nonresponsive cells in culture have significantly larger cytosolic pools of Etn and PEtn, it seemed possible that the cytosolic levels of Etn and/or PEtn in breast tumor specimens may be correlated with prognostic factors. Accordingly, we have examined the cytosolic contents of Etn and PEtn as well as some representative amino acids. The results indicate that the level of PEtn plus Etn and mitotic index are significantly correlated, and there is also a tendency of correlation between the level and the tumor grade according to the Page classification or the number of involved axillary nodes.

MATERIALS AND METHODS

Patients This study was carried out using tumor specimens excised from the primary sites of 53 patients with breast cancer who were operated at the National Cancer Center Hospital, Tokyo, Japan between August, 1988 and October, 1989. The tumor samples included various stages of the disease. The results of pathological analyses were available for most of the tumor specimens (unpublished results by T. Mita *et al.*). The range of patients' ages at the time of operation was from 25 to 72; 34% of the patients were premenopausal and 66% were post menopausal. Among the 53 specimens, 11 came with no data regarding the status of axillary nodes, mitotic indices or grading according to the Page classification.

Analysis of the cytosolic pool of amino acids and amines The cytosol was prepared from pulverized tissue (unpublished results by T. Mita *et al.*) and stored frozen at −70°C. Briefly, fat, necrotic and connective tissues were removed from the excised specimens, blood was washed away with cold saline, and the tissue was then pulverized in liquid N_2. The resulting powder was homogenized in 7 volumes of homogenization buffer consisting of 10 m*M* Tris-HCl, pH 7.4, 1 m*M* EDTA, 3 m*M* sodium azide, 12 m*M* monothioglycerol, and 10% glycerol in ice with a Polytron homogenizer using three 15 s pulses with 45 s intervals for cooling. The homogenate was centrifuged at 105,000*g* for 60 min at 4°C. The supernatant was stored frozen at −70°C until required for analysis.

An equal volume of cold methanol was added to the cytosol and centrifuged at 4°C for 10 min at 14,000 rpm in a microfuge. An appropriate amount of the resulting supernatant was derivatized and analyzed by high-performance liquid chromatography (HPLC) with electrochemical detection using a method developed by Ellison *et al.*[10] The derivatizing agent consisted of 50 mg *o*-phthaldialdehyde and 40 μl 2-mercaptoethanol in 1 ml of ethanol and 9 ml of 0.1 *M* sodium tetraborate decahydrate, pH 9.4. An appropriate amount of a sample was made up to 285 μl with methanol/H_2O (1:1 by vol), 65 μl of the derivatizing agent was added, and the mixture was vortexed immediately. The reaction mixture was incubated for exactly 2 min at room temperature, and 20 μl was injected onto a C-18 column (Beckman Instrument, Palo Alto, CA). A Toyo Soda HLC 803D with a Toyo Soda EC-8000 EC system was used for chromatography and detection. The mobile phase was 0.1 *M* sodium phosphate buffer, pH 5.2 with 37% methanol. Fifty to 500 pmol of standard amino acids or amines could be reproducibly quantitated by this method. A typical elution pattern of standard amino acids, PEtn, and Etn is shown in Fig. 1A. Both Etn and PEtn were eluted as distinct peaks and could easily be quantitated. The stability of derivatized individual amino acids and amines varied considerably. However, the chromatogram was highly reproducible as long as the duration of the derivatizing reaction and the running time of the column were kept constant. Amino acids and amines in the samples were identified by cochromatography with known standards and quantitated by comparing their peak heights with those of known standards, which were run at the beginning and the end of each day.

RESULTS

Characteristics of the assay system Typical chromatograms of standards and a breast tumor sample are shown in Fig. 1A and 1B. Major amino acids that can be derivatized and identified in tumor samples are indicated. Not all amino acids could be derivatized and identified by the present assay method, nor could the majority of identifiable amino acids be resolved as single peaks. Etn and PEtn were, however, eluted as distinct peaks. Major peaks in tumor samples were identified by cochromatography with standard amino acids as well as from the elution times, which were also reliable identification markers.

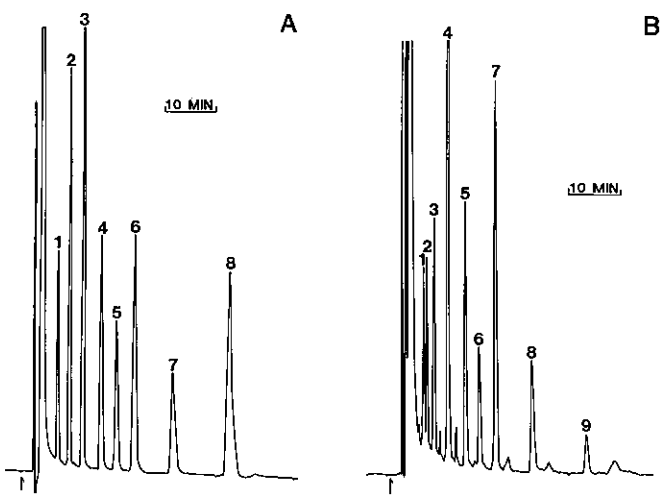

Fig. 1. Chromatograms of standards and a tumor sample. Samples were derivatized and chromatographed by HPLC with an EC detector as described in "Materials and Methods." The arrow indicates the injection time point. The numbers corresponding to the peaks represent the following amino acids and amines. A. 250 pmol each of 1, glutamic acid; 2, glutamine; 3, arginine; 4, glycine; 5, PEtn; 6, taurine; 7, alanine; 8, Etn. B. A tumor sample containing 4.6 μg of protein was analyzed. Under the conditions employed, PEtn and Etn were eluted as distinct peaks. However, alanine and tyrosine, glycine and threonine, glutamine and serine were co-eluted as single peaks, respectively. 1, glutamic acid and asparagine; 2, histidine; 3, glutamine and serine; 4, arginine and carnosine; 5, glycine and threonine; 6, PEtn; 7, taurine; 8 alanine and tyrosine; 9, Etn.

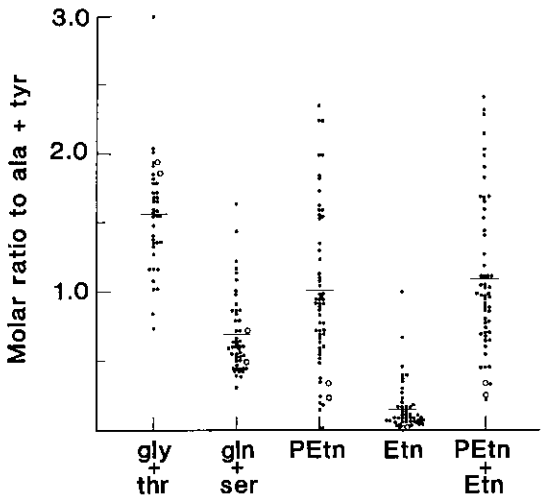

Fig. 2. Amino acids and amines in the cytosol of tumor samples. Derivatized cytosol of tumor and normal tissues was chromatographed as in Fig. 1. The heights of the peaks were used to calculate their molar amounts and the molar quantity of each peak divided by that of ala + tyr of the same sample was plotted. Closed circles represent tumor samples and open circles represent samples prepared from normal breast tissue enriched for epithelial cells. Bars indicate the means.

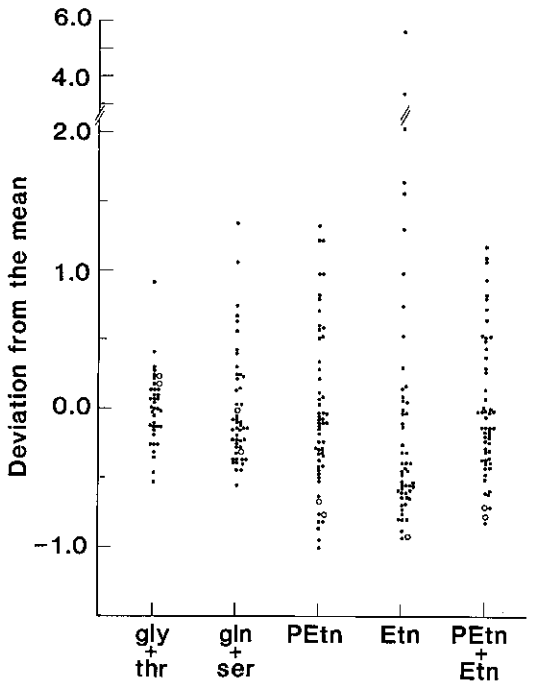

Fig. 3. Comparison of the deviations of the molar ratios of gly + thr, gln + ser, PEtn, Etn, and PEtn + Etn with respect to that of ala + tyr among tumor samples. For each value its difference from the mean was divided by the mean and the values thus obtained were plotted in order to compare the degree of variation among components which had different mean values. Standard deviation of gly + thr was 0.27 and that of PEtn was 0.58. ●, tumor sample; ○, normal sample.

The quantitation of eluted peaks was carried out using as references a set of chromatograms from standards run on the same day. The standard deviations of the peak heights of 250 pmol of Etn or PEtn analyzed over 6 separate days ranged from 3.7% to 5.4% of the mean values. Standard amino acids had similar deviations to PEtn or Etn. As long as the same, fresh (used within 12 days) batch of derivatizing agent was used, and the duration of the reaction and the elution rate were kept constant, the peak heights of all standards were essentially the same. In most cases a mixture containing 4 to 6 μg of cytosolic protein was analyzed per chromatogram.

As shown in Fig. 1B, all major components of tumor samples could be identified. The amount of these components per μg of cytosolic protein varied considerably depending on the sample, although separate analyses of the same sample gave reproducible results. For example, in extreme cases the variation among different tumor samples in the amount of alanine plus tyrosine could be 60 to 2300 pmol/10 μg protein, and generally if the level of one component in a sample was high, other components were also proportionally high. The reason for this variability is unknown. Based on the above observations, in order to compare the amount of PEtn and Etn among tumor samples the ratio of the molar amount of these components to that of alanine plus tyrosine (ala + tyr; these components co-eluted) was used (see Fig. 1).

PEtn, Etn, and amino acid levels among tumor samples

Quantitative analysis of glycine plus threonine (gly + thr; co-eluted as one peak), glutamine plus serine (gln + ser; co-eluted), PEtn, Etn, and PEtn plus Etn were thus carried out using their molar ratios to ala + tyr (sensitivities of detection of these amino acids are essentially the same) and the data were plotted (Fig. 2). The data include two samples of normal breast tissue. The gly + thr or gln + ser values among tumor samples seem to vary 2- to 3-fold less than those of PEtn, Etn, or PEtn + Etn. In order to better compare the distributions of these values, deviations from the mean divided by the mean $[(x_i\text{-}m)/m]$ were calculated and are plotted in Fig. 3. These results show firstly that the distribution of the values for PEtn, Etn or PEtn plus Etn are indeed significantly wider than those of amino acids. For example, the standard deviation of the deviation-from-the-mean-divided-by-the-mean for gly + thr was 0.27 and that for PEtn was 0.58, indicating that variability of the amounts of PEtn or Etn is greater than that of amino acids in tumor samples. Secondly, the major cytosolic

pool is PEtn rather than Etn, except in several samples where relatively high levels of Etn were found: in these samples PEtn levels were low, in contrast. The correlation between the levels of PEtn and Etn is shown in Fig. 4, where the values of PEtn/(ala+tyr) of each sample are plotted against those of Etn/(ala+tyr). No tumor samples contained high levels of both PEtn and Etn at the same time.

Prognostic factors and PEtn and Etn Distributions of the values for PEtn, Etn, and PEtn+Etn as well as those of representative amino acids were analyzed in relation to prognostic factors of the tumor specimens. Since some tumor specimens were accompanied with incomplete pathological data, the number of tumor samples which could be used for the study was less than the total number analyzed.

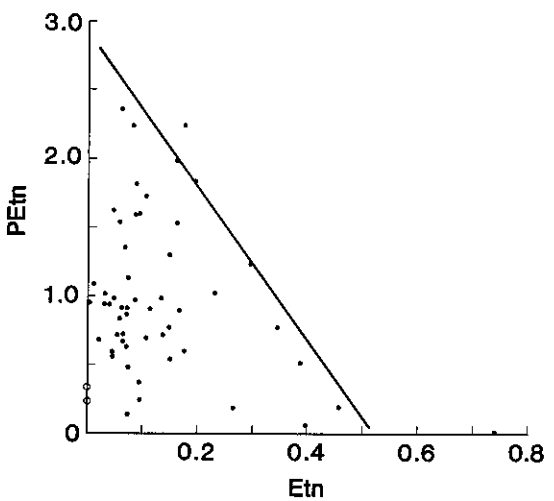

Fig. 4. Correlation between the contents of PEtn and Etn in individual tumor samples. The molar ratios of PEtn/(ala+tyr) were plotted against those of Etn/(ala+tyr). Each closed circle represents a tumor sample and open circles represent normal breast tissue.

Mitotic index: The values for gly+thr, gln+ser, PEtn, Etn, and PEtn+Etn were classified according to the frequency of mitotic indices as shown in Table I. The mean values of amino acids were found to be similar among groups having varied mitotic indices. However, when the mean values for PEtn alone or PEtn+Etn were compared, there was a tendency that the higher the mitotic index was, the higher the mean values of PEtn or PEtn+Etn, although the spread among samples was quite large. These values in normal samples were among the lowest. As shown in Table I, the PEtn or PEtn+Etn values of the 0–5 group were statistically significantly lower than in the groups having higher mitotic indices. When the samples were classified into two groups, 0 to 10 and 11+, instead of four, the mean values for PEtn were 0.81 ± 0.1 for the 0–10 group and 1.08 ± 0.16 for the 11+ group, indicating again that the samples having lower mitotic indices tend to have lower PEtn values. The PEtn+Etn values also showed a similar difference between the two groups. The mean Etn value was not significantly different between different groups.

Node status: Metastasis in axillary nodes was noted in about half of the patients analyzed. As indicated in Table II, the PEtn+Etn values were similarly distributed between positive and negative groups. However, when the PEtn+Etn values of the positive group were compared with the number of nodes that had metastasis, there was a tendency with moderate significance that the samples which had higher levels of the PEtn+Etn values were in groups having higher numbers of involved nodes (Table II). The PEtn values were distributed similarly to those of PEtn+Etn.

Table I. Mitotic Index and Cytosolic Levels of Amino Acids and Amines

Frequency of mitotic index	PEtn		PEtn+Etn		gly[a)]+thr		gln+ser	
	Number of samples	Mean	Number of samples	Mean	Number of samples	Mean	Number of samples	Mean
I: 0–5	14	0.59 ± 0.12[b)]	14	0.73 ± 0.10	8	1.58 ± 0.12	12	0.60 ± 0.07
II: 6–10	11	1.09 ± 0.13	11	1.20 ± 0.14	5	1.54 ± 0.10	9	0.74 ± 0.10
III: 11–20	13	0.96 ± 0.19	13	1.08 ± 0.18	10	1.51 ± 0.14	13	0.58 ± 0.04
IV: 21+	6	1.32 ± 0.31	6	1.45 ± 0.29	5	1.71 ± 0.08	5	0.74 ± 0.14
I vs. II	$P<0.01$		$P<0.01$		$0.5<P$		$0.2<P$	
I vs. III	$0.05<P<0.1$		$0.05<P<0.1$		$0.5<P$		$0.5<P$	
I vs. IV	$P<0.01$		$0.01<P<0.02$		$P=0.4$		$0.3<P$	
I vs. II+III+IV	$P<0.01$		$0.01<P<0.02$					

a) Abbreviations for amino acids are: gly, glycine; thr, threonine; gln, glutamine; ser, serine.
b) Standard error.

Table II. Node Status, Tumor Size and PEtn+Etn Values

	Number of samples	Mean		
Nodes				
I: +	25	1.13±0.12[a]	I vs. II	$0.5<P$
II: −	25	1.02±0.11		
Number of positive nodes				
I: 1–3	7	0.81±0.09	I vs. II	$P=0.3$
II: 4–9	10	1.03±0.16	II vs. III	$0.1<P<0.2$
III: 10+	8	1.50±0.27	I vs. III	$0.02<P<0.05$
Tumor size				
I: 1A	11	1.04±0.14		
II: 2A	26	1.09±0.10		
III: 3A	4	1.26±0.40		
IV: 4B,C	7	1.34±0.27	I vs. IV	$0.2<P<0.3$

a) Standard error.

Table III. Tumor Grade and Levels of Cytosolic Amino Acids and Amines

Page value	PEtn		PEtn+Etn		gly[a]+thr		gln+ser	
	Number of samples	Mean	Number of samples	Mean	Number of samples	Mean	Number of samples	Mean
1	7	0.76±0.23[b]	7	0.86±0.21	4	1.75±0.09	7	0.70±0.08
2	19	0.84±0.10	19	0.94±0.10	11	1.58±0.11	15	0.64±0.08
3	18	1.12±0.17	18	1.23±0.16	13	1.53±0.09	17	0.63±0.05
1 vs. 2	$0.5<P$		$0.5<P$		$0.3<P<0.4$		$0.5<P$	
2 vs. 3	$0.1<P<0.2$		$P=0.1$		$0.5<P$		$0.5<P$	
1 vs. 3	$0.2<P<0.3$		$0.2<P<0.3$		$0.2<P<0.3$		$0.5<P$	

a) Abbreviations for amino acids are as described in Table I.
b) Standard error.

Steroid hormone receptors: In this analysis, samples having more than 13 fmol of estrogen receptors and 10 fmol of progesterone receptors/mg cytosolic protein were regarded as positive. The mean values and distributions of the PEtn+Etn values were similar between estrogen and progesterone receptor-positive or -negative groups (1.19±0.11 vs. 1.00±0.09). The values for gly+thr, gln+ser, PEtn or Etn were also similar between the positive and negative groups (results not shown).

Page classification: The means of the gly+thr or gln+ser values were very similar among three groups, grades 1, 2, and 3, classified according to Page[2] (Table III). In the case of PEtn and Etn, although the difference was not statistically significant, the group of specimens classified as grade 3 tend to have higher values for PEtn, Etn, or PEtn+Etn (Table III).

Other histopathological indications: Histopathological parameters such as the degree of differentiation, tissue and cell atypia, and the tumor size were also available for many of the specimens tested. None of these properties appeared to be correlated with the PEtn, Etn or PEtn+Etn values except that the tumors whose size was large tended to have higher PEtn+Etn values, as shown in Table II.

DISCUSSION

The present study was carried out in order to explore the possibility that the cytosolic contents of two amines, PEtn and Etn, could be used as parameters for the assessment of breast tumors. The assay system employed is simple, reproducible and requires only a small portion of the cytosol prepared for steroid hormone receptor and other assays. A majority of the tumor samples contained significantly larger amounts of PEtn than Etn suggesting that minimal degradation took place during the preparation and storage of the cytosol.[1] Therefore, it is likely that the data obtained closely reflect the *in vivo* state of the tissue.

We found that the cytosolic contents of these amines were highly variable and moreover, the variation was significantly higher than those of amino acids. A positive correlation was observed between the PEtn and PEtn+Etn values and the mitotic index, and a similar tendency, although not statistically significant, was observed for clinical stages of the tumor and the tumor size. There was no apparent correlation between the PEtn or PEtn+Etn values and the steroid hormone receptor status or histopathological parameters such as the degree of differentiation or tissue and cell atypia. The PEtn+Etn values were similarly distributed between node-positive and negative groups. However, within the node-positive group, patients with higher numbers of involved nodes tended to have higher PEtn+ Etn values. This suggests that the ones having higher PEtn+Etn values among the node-negative group may have a potential to be highly malignant. There were five specimens that had PEtn+Etn values higher than 1.7 among the ones for which complete pathological data were available. It may be important to note that all five had mitotic index values higher than 10 (between 12 and 38), and four of them were node metastasis-positive and one negative. The tumor size of the negative one was the smallest (1A) at the time of operation. It may be of interest to determine whether the serum levels of these amines are elevated in patients with primary tumors as well as with distant metastasis.

The above results are consistent with the idea that during the progression of the breast tumor Etn-nonresponsiveness is acquired and these Etn-nonresponsive cells exhibit higher cytosolic levels of PEtn and/or Etn. Once the Etn-nonresponsiveness is acquired, the rate of cell proliferation may be accelerated since these cells are no longer dependent on the supply of these amines in the general circulation, which may be limiting for optimum growth. Analysis of a larger number of tumor specimens and clinical follow-up of the patients will be necessary to determine the significance and usefulness of these amines for the characterization of breast cancer. Further, it would be interesting to know whether a similar phenomenon can be found in other types of carcinomas. Normal cells of epithelial origin are generally Etn-responsive.[7] Therefore, it is possible that these cells undergo a process of malignant trransformation similar to that of mammary cells.

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