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Tissue Concentrations of Prothymosin Alpha: A Novel Proliferation Index of Primary Breast Cancer

Fernando Dominguez, Carlos Magdalena, Esperanza Cancio, Elena Roson, Jesus Paredes, Lourdes Loidi, Juan Zalvide, Maximo Fraga, Jeronimo Forteza, Benito J. Regueiro and Jose L. Puente

In 71 patents with classic invasive ductal carcinomas, levels of prothymosin alpha (PTα), as assayed by a radioimmunoassay that detects thymosin alpha 1 (the NH_2-terminal fragment of PTα), were significantly greater in tumour samples than in normal breast tissue. PTα levels were correlated with (a) the number of positive axillary lymph nodes ($r_s = 0.5384$, $P < 0.01$), and (b) the percentage of tumour cells in the S or G2/M phase as assessed by flow cytometry ($r_s = 0.5027$, $P < 0.01$). Since the beginning of this study in 1989, 21 patients have presented distant metastases, all of whom were previously shown to have tumour PTα levels greater than 124 ng of thymosin alpha 1/mg protein. The present report indicates that PTα might be used to identify breast cancer patients at high risk for distant metastases.
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INTRODUCTION

A MAJOR EFFORT in cancer research is aimed at finding new parameters that enable more precise means of identifying patients at high risk for local recurrences and distant metastases [1]. There are numerous indications that prolonged, increased cell proliferation is necessary for the development of tumours, particularly for hormonally related tumours, tumours secondary to various chemical exposures or virally related tumours (for a review see [2]). In breast cancer, cell proliferation indices have been shown to be prognosis-related [3].

Data gathered over the last few years strongly suggests that prothymosin alpha (PTα) expression may well be related to cell proliferation since (a) PTα mRNA levels were induced in serum-deprived fibroblasts 3T3 cells when they were stimulated to proliferate [4, 5] and PTα mRNA expression is also correlated to the proliferative activity of T-cells [6] and a small intestine-derived cell line [7]. (b) Immunohistochemical studies showed that PTα is expressed in proliferating but not quiescent cells in all tissues studied thus far [8–11]. (c) PTα mRNA antisense oligonucleotides were shown to inhibit cell division in myeloma cells [12]. (d) Activation of the transcription of the proto-oncogene *myc* led to a rapid increase in the transcription of the PTα gene, even in the absence of protein synthesis [13]. Although the mechanism of action of PTα yet awaits to be elucidated, present evidence suggests that it acts at a nuclear level since (i) it is synthesised without formation of a larger precursor polypeptide containing a hydrophobic signal sequence [14], (ii) Electron microscopy studies carried out in various tissues using immunogold techniques demonstrated a similar pattern of nuclear localisation irrespective of the tissue studied (11, [11]), and (iii) it contains a nuclear migrating consensus sequence [15].

In the present report the relationship between PTα levels and other parameters of clinical significance was studied in classic not otherwise specified (NOS) invasive ductal carcinomas [16] from affected patients at different clinical stages.

PATIENTS AND METHODS

Patients

Tumours were obtained from a series of 71 consecutive female patients with classic NOS invasive ductal carcinoma [16] who underwent definitive surgery at the Hospital General de Galicia (Santiago de Compostela, Spain) between 1989 and 1991. Patients with a second malignant neoplasm were excluded. The average age of patients was 55.5 years (range 31–94). Patients were divided into four stages, according to the following criteria: stage I, tumour ≤ 2 cm in diameter, limited to the breast and without positive nodes (16 patients); stage II, tumour ≤ 5 cm in diameter, or whatever size in the case of axillary metastasis (33 patients); stage III, tumour > 5 cm in diameter, or any size with advanced locoregional disease (20 patients); and stage IV, distant metastasis (2 patients).

PTα radioimmunoassay

Small slices (about 5 mg) of tumour and normal tissues obtained during surgery were homogenised with a Polytron homogeniser in phosphate buffered saline (PBS)–EDTA (PBS 0.05 mol/l, pH 7.5; EDTA, 2 mol/l) centrifuged at 14000 *g* for 15 min and the supernatant analysed for PTα and protein; a recovery test was carried out and it was found that more than 90% of the thymosin α 1 added was recovered in the supernatant. The radiolabelled ligand was [I^{125}]Tyr^0-thymosin α 1 and synthetic thymosin α 1 was employed to standardise the assay; the standard curve range was between 0.025 to 10 pmoles and the displacement obtained was ($X \pm$S.E.M.), ED-20 = 1.99±0.5; ED-50 = 0.15±0.014 and ED-80 = 0.028±0.0049. The antibody employed in the experiments reported here was raised against synthetic thymosin α 1 and recognises both thymosin α1 and PTα with a high specificity [8]. A sheep anti-rabbit IgG was used as a second antibody to separate bound and free ligand. The bound ligand was collected by centrifugation in the presence of 10% polyethylene glycol. Tissue homogenates were prepared at room temperature in order to convert tissue PTα to thymosin α 1 [8, 17]; therefore, the results are expressed as thymosin α 1 equivalents.

To assess the effect that room temperature incubations have on thymosin α 1 levels, we incubated radioactive thymosin α 1 with homogenates from various tumours at room temperature for 0, 2, 4, 6 and 24 h; then 5-μl aliquots from the incubation mixture were spotted on filters and washed twice in cold 10% trichloroacetic acid, 5% trichloroacetic acid and absolute ethanol and the radioactivity remaining in the filters was counted. Up to 20% differences were found between the amount of radioactive thymosin α 1 detected after 0 and 24 h. We therefore concluded that manipulations of the samples at room temperature did not substantially modify the apparent tumour thymosin α 1 content.

In order to investigate whether immunoreactive materials other than thymosin α 1 were present in tumour samples, we fractionated a tumour homogenate on a Sephadex G-50 column and the resulting fractions were assayed for thymosin α 1 immunoreactivity. We found only one immunoreactive peak, which coeluted with thymosin α 1.

Immunohistochemistry

Tumour serial sections were alternately stained with haemotoxylin–eosin and with IgG fractions purified from anti-thymosin α 1 antiserum as described [8].

Flow cytometry

Tumour samples were resected and processed on a single day. Fresh tumours were mechanically dispersed and the cell processing was done according to Vendelov and Christensen [18] with minor modifications [5].

Statistical analysis

Wilcoxon test was used for paired samples and Mann–Whitney test was used for unpaired samples. Correlations are expressed as Spearman rank correlation coefficients (r_s).

The association of PTα levels with other clinicopathological factors was assessed by the χ^2 test. The use of a cutoff level for PTα levels produced lower P values than the use of continuous variables, after testing various cutoff levels we found that the value of 124 ng of thymosin α 1/mg of protein gave lower P values; therefore, patients were divided into two groups: (a) <124 ng/mg protein and (b) >124 ng/mg protein. All data were analysed with the use of the Statgraph statistical package (Statistical Graphic Co., Rockville, Massachusetts, U.S.A.).

RESULTS

Expression of PTα in tumour cells

PTα levels in tumour samples were significantly greater ($P < 0.0001$) than PTα levels found in adjacent normal breast tissue samples obtained from the same patient (Fig. 1).

By means of immunohistochemical techniques, we confirmed that tumour cells were expressing PTα (Fig. 2).

Correspondence to F. Dominguez.
F. Dominguez, L. Loidi and J. Zalvide are at the Departamento de Fisiologia, Laboratorio de Neurociencia "Ramon Dominguez"; C. Magdalena, J. Paredes and J. L. Puente are at the Departamento de Cirugia; E. Cancio and B. J. Regueiro are at the Departamento de Microbiologia; E. Roson is at the Departamento de Ciencias Morfologicas; and M. Fraga and J. Forteza are at the Servicio de Anatomia Patologica, Hospital Xeral de Galicia, Facultad de Medicina, 15705 Santiago de Compostela, Spain.
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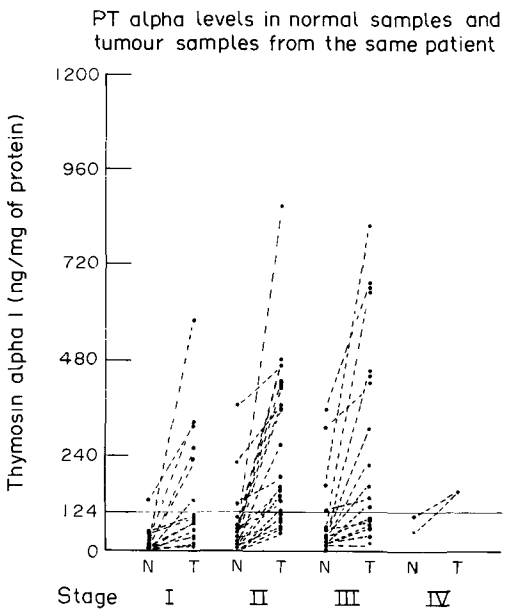

Fig. 1. Relationship of PTα levels in normal (N,●) and tumour (T, ○) breast tissue from each of the 71 patients classified according to clinical stage (I–IV, see Patients and Methods).

PTα levels, patient's age and tumour size

In the present report we found no significant correlation between PTα levels and patient's age ($r_s = -0.1243$, $P = 0.3161$) or tumour size ($r_s = 0.1077$, $P = 0.3852$).

PTα levels and positive axillary lymph nodes

Nodal involvement was histologically determined. PTα levels in breast cancers with nodal metastases were significantly higher than in those without lymph node involvement (median, interquartile range 337.9, 397.4 vs. 106.9, 245.5 ng of thymosin α 1/mg protein, $P < 0.01$). Moreover, the number of positive nodes correlated strongly with tumour PTα levels ($r_s = 0.5384$, $P < 0.01$).

The use of commonly applied cutoff levels for the number of affected nodes produced lower P values than the use of continuous variables; thus, 71 patients were divided into four groups according to the following criteria: (1) patients without positive nodes (28 patients); (2) patients with one to three positive nodes (18 patients); (3) patients with four to seven positive nodes (11 patients); and (4) patients with more than seven positive nodes (14 patients). Cross-tabulation of patients according to PTα levels by the number of positive nodes is shown in Table 1. An association between both parameters was found as estimated by χ^2 analysis ($\chi^2 = 13.02$, degrees of freedom = 3, $P < 0.005$, Kendall's Tau $B = 0.3859$, significance = 0.00041).

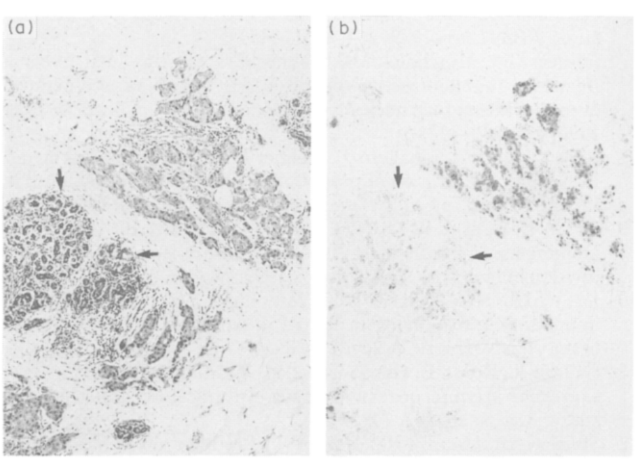

Fig. 2. Proximate sections of a classic (NOS) invasive ductal carcinoma stained with haematoxylin–eosin (a) and immunohistochemical methods using IgG fractions purified from rabit anti-thymosin α 1 polyclonal antiserum (b). Intense immunoreactivity is visible in numerous tumour cells. In contrast, normal breast (arrows) showed minimal positivity. Original magnification 90×.

Table 1. PTα levels in relation to axillary lymph node status

	Number of positive nodes			
	0	1–3	4–7	>7
A*	16	5	2	1
(PTα<124)	(57%)	(26%)	(18%)	(7%)
B	12	14	9	13
(PTα>124)	(43%)	(74%)	(82%)	(93%)

Number of patients (percentage). *PTα levels less than (A) or greater than (B) 124 ng of thymosin α 1/mg protein.

PTα levels and tumour histological grade

Tumours were divided according to the Bloom and Richardson method as described [19]. 82.6% (19 out of 23) of the tumours with a histological grade 3 had PTα levels greater than 124 ng of thymosin α 1/mg protein vs. 57.1% and 58.3% of tumours with histological grades 2 and 1, respectively. Moreover, patients with a histological grade 3 had PTα levels greater than those with histological grades 1 and 2 (median, interquartile range 523.9, 552.1 vs. 170.8, 231.4 ng of thymosin α 1/mg protein respectively, $P < 0.001$). The fact that grade 3 tumours have the greatest PTα levels is not unexpected based on the fact that PTα expression has been linked to cell proliferation and the number of mitosis is a major criterion in the grading system [19].

PTα levels and tumour cytological type

Tumours were divided into cytological types (A, B, AB and C) according to Dawson's criteria [20]. It is noteworthy that 81% of tumours of cytological type B and 69% of type AB had PTα levels greater than 124 ng of thymosin α 1/mg protein. Conversely, 42 and 43% of the tumours graded A and C had respective PTα levels greater than 124 ng of thymosin α 1/mg protein (Table 2). Both type A and C are indicative factors of a higher probability of long-term survival, while either type AB or B has a relatively lower probability of 25-year survival [20].

Table 2. PTα levels in relation to tumour's cytological type [20]

	Cytological type			
	A	B	AB	C
A*	7	6	5	4
(PTα<124)	(58%)	(19%)	(31%)	(57%)
B	5	26	11	3
(PTα>124)	(42%)	(81%)	(69%)	(43%)

Number of patients (percentage). *PTα levels less than (A) or greater than (B) 124 ng of thymosin α 1/mg protein.

PTα levels and tumor DNA flow cytometry

Tumours with PTα levels greater than 124 ng of thymosin alpha 1/mg protein had a significantly lower percentage of cells in the G0/G1 phase than those tumours with PTα levels lower than 124 ng of thymosin α 1/mg protein ($P < 0.05$). Actually, a significant negative correlation was found between PTα levels and the percentage of cells in the G0/G1 phase ($r_s = -0.52$, $P < 0.01$). On the other hand, a positive correlation was found between the percentage of cells in the S+G2/M phase and PTα levels ($r_s = 0.5027$, $P < 0.01$). However, no correlation was found between PTα levels and the S phase or the G2/M phase when they were assessed separately.

PTα levels, local recurrences and distant metastasis

After a follow-up ranging between 6 and 30 months after surgery, 71 patients were divided into three different groups (a) patients without local recurrences or distant metastasis (L) patients presenting local recurrences and (M) patients with distant metastasis. PTα levels in group M were significantly greater than PTα levels in either group A or L ($P < 0.0001$ and $P < 0.05$, respectively). 12.5% of stage I patients developed local recurrences or distant metastasis vs. 39.4% of stage II patients, 55% of stage III and 100% of stage IV. All patients presenting distant metastasis had PTα levels greater than 124 ng of thymosin α 1/mg protein (Table 3). By means of χ^2 analysis a significant association was shown between the number of patients with distant metastasis and PTα levels greater than 124 ng of thymosin α 1/mg protein ($\chi^2 = 13.158$, degrees of freedom = 1, $P < 0.0003$ with Yates correction, two-tail Fisher's exact test $P < 0.0003$).

DISCUSSION

PTα is present only in the proliferative cycle including the final steps of G_1 phase, throughout the S and G_2 phases, and in initial steps of prophase, but is not expressed in non-proliferating cells [9]. It has been postulated that PTα might be involved in DNA replication [9], suggesting that PTα may play a basic role in normal cells. This led us to postulate that the assay of PTα levels might be used to estimate the proliferating activity of human tumours. Our present data support this hypothesis, and concur with previous findings showing that PTα mRNA was elevated in leukaemic cells [6]. Our present data is also in agreement with previous reports that stress the importance of proliferation indices in the prognosis of breast tumours [1, 21]. We found that PTα levels could be used as a marker of the potential malignancy of breast tumours. Support to our claim comes from: (a) the well-established fact that axillary lymph node involvement is indicative of tumour capacity for direct dissemination; increasing numbers of positive nodes are associated with an increasingly poor prognosis [22]. We found a significant correlation between tumour PTα levels and the number of positive nodes. (b) 82% of the histological graded 3 tumours had high PTα levels; (c) 81% of the histological graded B tumours (i.e. those with the lowest odds of long-term survival [20]) had high PTα levels; (d) a significant correlation was found between PTα levels and the proportion of proliferating tumor cells, as assessed by flow cytometry; and (e) of special relevance is the fact that all patients who developed distant metastasis had high PTα levels and that a correlation between these parameters was demonstrated.

We do not believe that PTα expression is a specific feature of malignancy, since PTα is also found in normal tissues, but a measure of the proliferating activity of the tumour. In the present report we found that tumour PTα levels might help to identify stage I patients at high risk for distant metastasis. However, further studies with a larger series of patients and a longer follow-up period are now needed. Finally, we would like to draw attention to the fact that PTα determination might be helpful in studying the proliferating activity of other cancers since, to our knowledge, all tissues express PTα and PTα expression is restricted to actively proliferating cells [8–11]. Our study represents a preliminary attempt to elucidate the possible application of PTα as a cell proliferation marker in human cancers.

Table 3. PTα levels in relation to local recurrences and distant metastasis

	Local recurrences/distant metastasis		
	Neither	Local recurrences	Distant metastasis
A* (PTα<124)	22 (51%)	2 (29%)	0 (0%)
B (PTα>124)	21 (49%)	5 (71%)	21 (100%)

Number of patients (percentage). *PTα levels less than (A) or greater than (B) 124 ng of thymosin α 1/mg protein.

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EO9: A Novel Bioreductive Alkylating Indoloquinone With Preferential Solid Tumour Activity and Lack of Bone Marrow Toxicity in Preclinical Models

H.R. Hendriks, P.E. Pizao, D.P. Berger, K.L. Kooistra, M.C. Bibby, E. Boven, H.C. Dreef-van der Meulen, R.E.C. Henrar, H.H. Fiebig, J.A. Double, H.W. Hornstra, H.M. Pinedo, P. Workman and G. Schwartsmann

EO9 is a novel and fully synthetic bioreductive alkylating indoloquinone. Although structurally-related to mitomycin C, EO9 exhibits a distinct preclinical antitumour profile and there are also differences in its biochemical activation. In this study, EO9 was found to demonstrate preferential cytotoxicity against solid tumours *in vitro* as compared to leukaemia cell lines both in the Corbett two-tumour assay and in the disease-oriented human tumour cell line panel of the U.S. National Cancer Institute. In the latter system activity was particularly apparent in colon, melanoma and central nervous system lines, together with some renal and non-small cell lung lines. Preferential cytotoxicity towards hypoxic versus aerobic EMT6 mouse mammary tumour cells was observed. *In vivo*, EO9 was inactive against the P388 murine leukaemia, while exerting significant antiproliferative effects against several murine and human solid tumours, including the generally resistant MAC mouse colon tumours and gastric, ovarian and breast xenografts. These results confirmed *in vitro* observations of preferential solid tumour activity. In animal toxicology studies, EO9 induced vascular congestion in the gastrointestinal tract, but no significant bone marrow toxicity. The LD_{10} value of EO9 after a single intravenous injection into mice was 9 mg/kg (27 mg/m^2). A dose of one-tenth of the mouse equivalent LD_{10} (2.7 mg/m^2), the recommended starting dose for clinical phase I studies, was found to be safe in rats. Considering its distinct mechanism of bioactivation as compared to mitomycin C, its preferential solid tumour activity, its excellent activity against hypoxic cells, and lack of significant bone marrow toxicity in animal studies, EO9 has been selected for clinical evaluation within the framework of the EORTC.
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INTRODUCTION

EO9 [3-HYDROXY-5-AZIRIDINYL-1-methyl-2-(1H-indole-4,7-dione)-propenol] (E85/053, NSC 382459) is the lead compound in a series of novel and fully synthetic bioreductive alkylating indoloquinones, originally synthesised by Oostveen and Speckamp [1]. Although structurally related to mitomycin C (MMC) (Fig 1), preliminary *in vitro* evaluation of EO9 has suggested that this compound differs from MMC in its antitumour profile [2]. There are also differences in the mechanism of biochemical activation. In contrast to MMC, EO9 was shown to function *in vitro* as an excellent substrate for reduction by human and murine DT-diaphorase (DTD) at physiological pH [3, 4]. Experiments performed with purified rodent DTD have shown that EO9 reduction by this enzyme prompted the formation of bioactivated