The Activity of Thymidine Phosphorylase Correlates with Tumor Size and Lymph Nodes Status in Breast Carcinoma

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The platelet-derived endothelial cell growth factor (PD-ECGF) is one of the potent angiogenic factors. Recently, its homology with thymidine phosphorylase (dThdPase), an enzyme involved in pyrimidine nucleoside metabolism, has been shown. In the present study, dThdPase activity was evaluated spectrophotometrically in 43 breast carcinomas and in 19 cases of non-neoplastic breast tissues. The mean dThdPase activity in breast cancer was almost six fold higher than in normal, non-neoplastic breast tissues (1.92 and 0.29 μmol thymine (T) × mg prot. $^{-1}$ × h $^{-1}$ respectively). The enzyme activity significantly correlated with axillary lymph node status ($p = 0.0076$) and with tumor size ($p = 0.0099$). Besides, the intratumoral microvessel density (MD) was evaluated using the CD 31 mouse anti-human monoclonal antibody, and there was no correlation between the level of enzymatic activity and a number of microvessels. The positive significant correlation of thymidine phosphorylase activity with prognostic factors in breast cancer patients with no relation to the number of microvessels needs further examination to confirm the prognostic significance of the level of dThdPase.

Introduction

Angiogenesis plays a crucial role in tumor progression and metastases formation in many of human malignancies. There is a lot of evidence that microvessels density is an independent prognostic factor in gastrointestinal tumors (Chung *et al.*, 1996), endometrial carcinoma (Kaku *et al.*,1997) and breast carcinoma (Gasparini *et al.*,1995). The starting point for the formation of the vasculature is endothelial cell differentiation, proliferation, and finally organisation into the blood islands, and vascular structures. These phenomena are regulated by a paracrine system, which includes several angiogenic and anti-angiogenic peptides.

Platelet-derived endothelial cell growth factor (PD-ECGF) was described as a potent angiogenic factor present in platelets (Ishikawa *et al.*, 1989). PD-ECGF has chemotactic properties for endothelial cells *in vitro* and angiogenic activity *in vivo* (Folkman, 1996; Takayashi *et al.*, 1996). PD-ECGF expression correlated well with an intensity of angiogenesis in breast carcinoma (Relf *et al.*, 1997). Recently, the homology between PD-ECGF and thymidine phosphorylase (dThdPase) has been demonstrated (Moghdam *et al.*, 1995; Takebayashi *et al.*, 1996).

Thymidine phosphorylase is an enzyme involved in pyrimidine nucleoside metabolism. Human dThdPase catalyses the reversible phosphorolysis of the thymidine and other pyrimidine-2'-deoxyribosides. Pauly demonstrated elevated levels of plasma concentration of dThdPase in breast cancer patients in comparison with healthy volunteers (Pauly *et al.*, 1977). Similarly, almost ten-fold overexpression of dThdPase in human tumors compared to normal tissues was shown (Moghdam *et al.*, 1995; Takebayashi *et al.*, 1996; Toi *et al.*, 1995). Furthermore, it seems that overexpression of dThdPase correlates with microvessels density in breast carcinoma (Toi *et al.*, 1995) and intensity of angiogenesis in colon carcinoma (Folkman, 1996; Takayashi *et al.*, 1996; Takebayashi *et al.*, 1996).

In this paper we studied a correlation between enzymatic activity of dThdPase and clinical data (lymph node status, tumor size, and carcinoma

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grade), and with microvessels density in breast carcinoma.

Lymph node status, tumor size and histological grade are factors of the most important prognostic significance in breast carcinoma.

Material and Methods

Breast tissue

Breast cancer tissue was obtained from 43 patients undergoing mastectomy. Tissue samples (approximately 0.5 cm^3) were dissected from tumors immediately following surgery and frozen in low temperature (−80 °C) until required. Besides, in 19 cases, normal breast tissues (non-tumoral) were obtained at the time of mastectomy. All these samples were histologically proven to be non-neoplastic.

All patients (except one) underwent modified radical mastectomy with a full axillary lymph nodes dissection. One patient was subjected to a simple mastectomy. Every case was divided into classes depending on tumor size – < 2 cm. and > 2 cm. Besides, the number of involved lymph nodes was coded as class I – no metastases, class II – 1–3 metastases and class III > 3 metastases.

Histology and immunohistochemistry.

For the routine purposes, hematoxilin and eosin staining were performed. Histological diagnoses were done according to the WHO criteria using Bloom-Richardson grading system for the Ductal Carcinoma NOS (not otherwise specified) (Bloom and Richardson, 1957).

For the immunohistochemistry staining, routinely processed, formalin-fixed, paraffin embedded, tissue blocks were cut on silanized slides. Microvessel assessment was performed using a mouse anti-human CD 31 (Dako) antibody in dilution 1:40 with streptavidin-biotin technique following prolonged (13 min) enzymatic digestion with trypsin. Microvessel counting, according to the Weidner method (Vermeulen *et al.,* 1996), was initiated in those areas of most intensive vascularization (hot-spots) identified by scanning the specimen at low power magnification. Counting was continued in ten consecutive high power fields (400X) and was performed independently by two pathologists [R. K.,D.J-K.]. In cases of more than 15% discrepancy, two pathologists repeated counting simultaneously, and the consensus result was obtained. Any positive staining cell or cells cluster (with or without lumen) was included into a scoring. As the scoring result, a median per one high power field was calculated.

Enzyme assay

Preparation of the cytosol fraction

Tissue samples were homogenised in a glass homogenizer in 4 vol. of ice cold buffer (1 mM EDTA, 0.02% mercaptoethanol, 2 mM phenylmethanesulfonyl fluoride [PMSF], 10 mM tris (hydroxymethyl) aminomethane – maleate, pH 6,5) with 10% glycerol and then centrifuged at $100\,000 \times g$ for 1 h, to obtain cytosol fraction. It was then pooled and directly taken for analysis.

Enzyme assay

dThdPase activity was assessed by the spectrophotometric method described by Yoshimura (Yoshimura *et al.,* 1990) in our own modification, using the formation of thymine (T) from thymidine in the presence of arsenate. The incubation mixture of 0.5 ml final volume contained 0.1 M tris (hydroxymetyl) aminomethane – arsenate buffer (pH 6.5), 10 mM dThd and cytosol fraction. After a 1 – h incubation at 37 °C, the reaction was stopped by adding 0.5 ml 1M NaOH and the thymine formed was measured with absorbance at 300 nm. The protein content was determined according to the method described by Bradford (1976).

One unit of activity of dThdPase was defined as the amount of the enzyme which was required to form 1μM of free base per 1 hr. Specific activity was defined as the number of the enzyme activity units per milligram of protein.

Statistical analysis

Statistical analysis was performed using Kruskal-Wallis ANOVA, the median test and Spearman range correlation to evaluate the significance of the differences. The $p < 0.05$ was taken as the level of statistical significance.

Results

Activity of dThdPase

The activity of dThdPase measured as μmol of T × mg prot. $^{-1}$ × h $^{-1}$ in normal tissues (19 cases) ranged from 0.10 to 0.64. The mean activity in 19 cases of the normal breast tissue was 0.29 μmol of T × mg prot. $^{-1}$ × h $^{-1}$ (Table I). The mean enzyme activity in non-neoplastic tissue (range from 0.80 to 4.38 μmol of T × mg prot. $^{-1}$ × h $^{-1}$) was significantly lower ($p < 0.00005$) than the activity of dThdPase in breast cancer tissue, where the mean activity was 1.92 μmol of T × mg prot. $^{-1}$ × h $^{-1}$.

The positive, significant correlation ($p < 0.0076$) was observed when dThdPase activity and lymph node status were compared. When the lymph

Table I. Clinical characteristics of studied cases, dThdPase activity and microvessels count.

Case	Histological type & grade	Number of involved lymph nodes	Tumor size [mm]	dThdPase activity*		Microvessels count **
				Normal tissue	Tumor	
1	IDC II	0	18		0.94	22.3
2	IDC III	0	45		2.42	NE***
3	IDC II	0	20		1.72	12.1
4	IDC II	0	23		2.24	NE
5	IDC II	0	19	0.10	2.71	15.9
6	IDC I	0	20		1.49	24.3
7	IDC III	0	30		2.48	20.8
8	IDC II	0	15		1.38	11.9
9	IDC III	0	18	0.27	2.32	14.9
10	IDC III	SM****	25		2.34	14.3
11	IDC II	0	22		1.37	18.6
12	IDC III	0	32	0.22	1.87	11.4
13	IDC I	0	18	0.29	0.87	21.1
14	ILC	0	22	0.64	1.69	25.6
15	IDC III	0	18	0.31	1.29	34.9
16	IDC III	0	17		0.90	20.7
17	IDC III	0	22		1.84	11.8
18	ILC	0	17	0.39	1.75	3.2
19	IDC III	0	60	0.17	1.75	11.8
20	IDC III	0	10		1.83	19.1
21	ILC	0	12		0.80	NE
22	IDC I	0	10		1.72	13.5
23	ILC	0	32	0.21	1.32	20.5
24	IDC II	0	15		1.34	19.7
25	ILC	1	24		0.91	11.9
26	IDC II	1	25		2.27	NE
27	IDC II	1	35	0.17	2.27	NE
28	IDC II	1	23	0.46	2.43	18.7
29	IDC II	1	30	0.51	2.11	29.7
30	IDC III	1	11		1.58	19.8
31	ILC	1	20	0.22	1.83	31.2
32	IDC III	2	32		1.95	20.7
33	IDC III	2	35		2.49	18.7
34	IDC III	2	35		2.51	NE
35	IDC III	2	19	0.39	1.69	25.0
36	IDC II	3	30		3.20	14.3
37	IDC II	3	18		2.21	16.4
38	IDC III	3	22		2.23	20.4
39	ILC	4	22	0.32	3.75	23.2
40	IDC II	5	11	0.21	4.38	12.3
41	IDC II	5	22	0.27	2.41	15.7
42	IDC III	8	25	0.17	1.17	26.9
43	IDC III	22	30	0.46	1.83	17.0

IDC – Infiltrating Ductal Carcinoma, ILC – Infiltrating Lobular Carcinoma.
* – dThdPase activity measured in μmol of thymine (T) × mg prot. $^{-1}$ × h $^{-1}$.
** – number of microvessels per 1 high power field (microscope magnification 400 ×).
*** – not examined.
**** – simple mastectomy.

nodes metastases were scored into 3 classes, the thymidine phosphorylase activity raised with the score of involved lymph nodes (Fig. 1).

Similar results were obtained for the correlation of dThdPase activity and a tumor size. The association between maximal diameter of the tumor and the enzyme activity was statistically significant – $p = 0.0089$. When the tumors were stratified into two groups: less than 20 mm, and more than 20 mm, the results were also significant ($p = 0.0099$) (Fig. 2).

We found no correlation between the dThdPase activity and histological grade of breast cancer. Most of our patients (36) had Infiltrating ductal carcinoma NOS (IDC). When only IDC cases were analyses in regards to the Bloom and Richardson histological grade, the activity of the enzyme was higher in a grade II in comparison with a grade I, but this correlation was not statistically significant – p = 0.3299 (Fig. 3).

Microvessels density (MD)

Assessing the MD in the tumor, we found no significant correlation between MD and enzyme activity, tumor size and lymph nodes status.

Discussion

Since the time when Folkman suggested, that tumor growth was dependent on angiogenesis, the study of assessing angiogenesis and its regulatory mechanism has been under intensive investigation.

Several angiogenic and anti-angiogenic factors are currently known, and platelet-derived endothelial cell growth factor (PD-ECGF) is one of them. PD-ECGF stimulates the growth and chemotaxis of endothelial cells *in vitro* and possesses angiogenic activity *in vivo* (Ishikawa *et al.*, 1989). Thymidine phosphorylase, which shows partial sequence homology with PD-ECGF (Furukawa *et al.*,

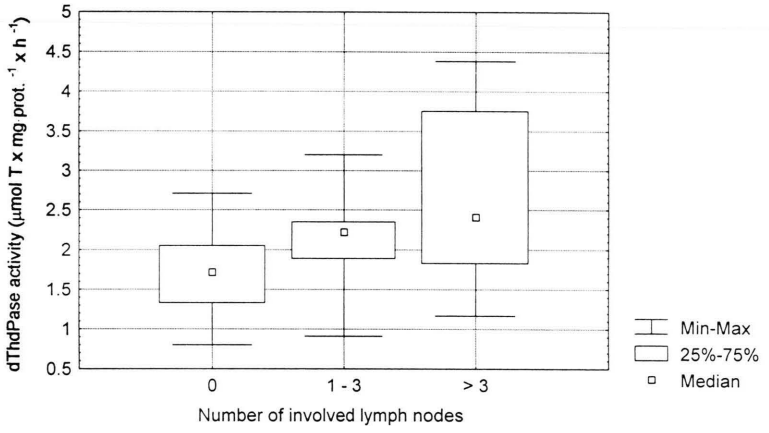

Fig. 1. dThdPase activity depending on lymph node staus. Spearman rank correlation. $R = 0.4015$, $p = 0.0076$.

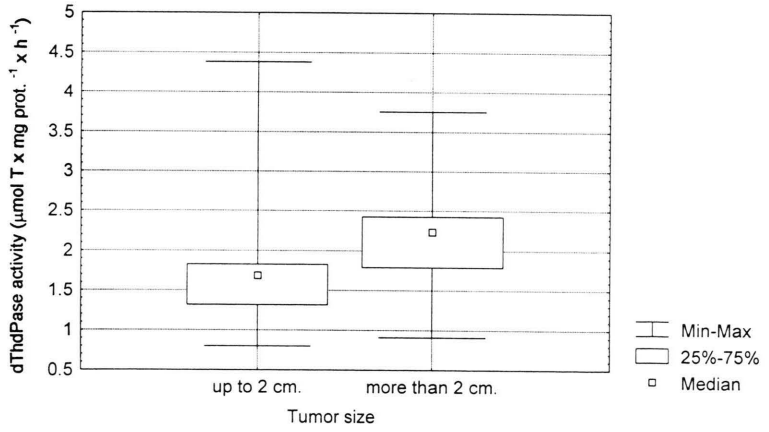

Fig. 2. dThdPase activity depending on tumor size. Kruskal-Wallis test. $p = 0.0099$.

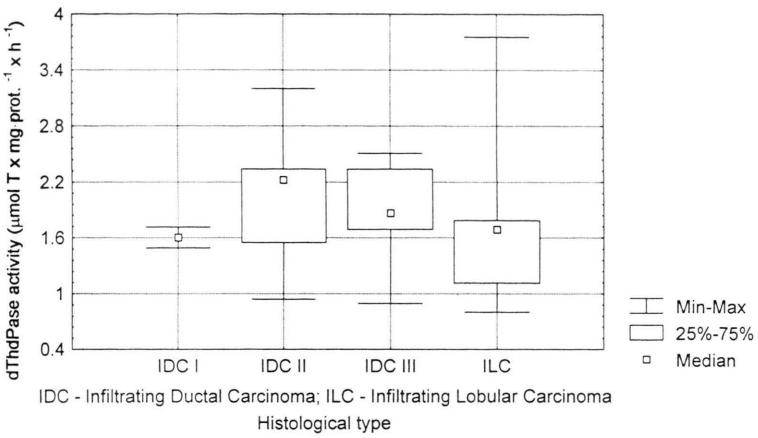

Fig. 3. dThdPase activity depending on histological type and grade of carcinoma. $p = 0.3299$.

1992), is an enzyme involved in the pyrimidine nucleotide metabolism (Moghdam *et al.*, 1995).

In most recent studies to investigate the expression of PD-ECGF/dThdPase in histological sections, immunohistochemical methods were applied (Takayashi *et al.*, 1996; Moghdam *et al.*, 1995; Toi *et al.*, 1995; Maeda *et al.*, 1997; Giatromanolaki *et al.*, 1997), but merely, a few authors assessed the activity of dThdPase using spectrophotometric methods (Kubota *et al.*, 1997). Both methods show upregulation of dThdPase expression/activity in cancer tissues in comparison with adjacent, non-neoplastic tissues (Takebayashi *et al.*, 1996). Analogously, we also demonstrated nearly six-fold higher enzyme activity in the tumor tissue.

The fact, that the dThdPase could exert a stimulatory effect on endothelial cells, raised the possibility, that dThdPase activity may be a novel prognostic factor. However, no correlation between dThdPase activity and prognosis in non-small cell lung cancer (Giatromanolaki *et al.*, 1997) or primary breast carcinoma (Toi *et al.*, 1995; Fox *et al.*, 1996) was detected. Furthermore, Fox studying lymph node-positive breast tumors also found no differences, in relapse-free and overall survival in respect to the dThdPase expression (Fox *et al.*, 1997). On the other hand, Takebayashi studying gastrointestinal and lung cancers, showed a higher enzyme activity in more advanced stages (Takebayashi *et al.*, 1996). Similar results were obtained in bladder cancer by Kubota (Kubota *et al.*, 1997). Thus, the issue is not settled.

In our study, an association between the level of dThdPase activity and clinical features such as axillary lymph node status and a tumor size was shown. These results may reflect prognostic significance of dThdPase, both in node-negative and node-positive breast carcinomas, but it clearly needs further investigation. The level of the activity of dThdPase should be further correlated with relapse-free and overall survival.

The majority of papers pointed-out to the correlation between the level of dThdPase activity and a number of intratumoral microvessels in breast cancer patients (Relf *et al.*, 1997; Toi *et al.*, 1995; Toi *et al.*, 1996). Only Fox failed to show such a correlation (Fox *et al.*, 1996). An absence of such a correlation in our studies is probably due to the small number of cases. Fox suggested, that dThdPase is important for remodelling of vasculature only in an early stage of tumor development, because the chemotactic, not mitogenic properties of dThdPase (Fox *et al.*, 1996). Moreover, Gasparini and Harris, reviewing the clinical importance of angiogenesis in breast cancer, found that prognostic significance of microvessels density was disclosed in early-stage breast carcinomas (Gasparini and Harris, 1995). However, other investigation (Folkman, 1996; Toi *et al.*, 1995) suggested, that the level of dThdPase activity is associated with the activity of various cytokines and growth factors like interleukin – 1 (IL-1), tumor necrosis factor α (TNF-α), interferon γ or basic fibroblast growth factor (β-FGF). Perhaps, in more advances stages of cancer these factors may play a more important role in a stimulation of the level of dThdPase, which in turn does not influence microvessels count.

Furthermore, apart from tumor cells, the expression of the dThdPase is exhibited in a variety of tumor infiltrating cells including macrophages or lymphocytes (Takebayashi *et al.,* 1996). It is unclear, however, whether this stimulatory pathway is really involved in tumoral angiogenesis.

We also investigated the putative association between intratumoral MD and both, lymph node status and tumor size. However, we showed no such a correlation, there several lines of evidence point to the MD as a prognostic factor in breast carcinoma (Obermair *et al.,* 1995; Bevilacqua *et al.,* 1995). In a study on 130 cases of infiltrating ductal carcinoma Aranda revealed no correlation with lymph node status (Aranda and Laforga, 1996). Similarly, Ravazoula found no association between MD and a number of metastases to axillary lymph nodes (Ravazoula *et al.,* 1996). Costello studied exclusively the lymph-node-negative breast cancer patients, showed no association of MD with clinical outcome and prognostic features such as tumor grade, and size, estrogen receptor status and accumulation of p53 protein (Costello *et al.,* 1995).

These conflicting results between possible prognostic significance of dThdPase and lack of its correlation with MD maybe due to relatively high heterogeneity in MD assessment. An international consensus on the methodology and criteria of evaluation of angiogenesis was suggested (Vermeulen *et al.,* 1996) but many problems have still appeared during a tumor sampling. DeJong warned against the heterogeneity in MD between different areas from the same section, between corresponding areas in different sections and between different blocks from the same tumor (De Jong *et al.,* 1995).

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