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Original Paper

The Activity and Expression of Thymidine Phosphorylase in Human Solid Tumours

Y. Takebayashi,[1,2] K. Yamada,[2] K. Miyadera,[1,3] T. Sumizawa,[1] T. Furukawa,[1] F. Kinoshita,[2] D. Aoki,[2] H. Okumura,[2] Y. Yamada,[3] S.-i. Akiyama[1] and T. Aikou[2]

[1]Department of Cancer Chemotherapy, Institute for Cancer Research; [2]First Department of Surgery, Kagoshima University, Sakuragaoka 8-35-1, Kagoshima 890; and [3]Taiho Pharmaceutical Co Ltd, Saitama 357, Japan

Thymidine phosphorylase (dThdPase) is identical to platelet-derived endothelial cell growth factor (PD-ECGF) and has angiogenic activity. Since dThdPase seems to have an important role in angiogenesis of tumours, we measured the activity and expression of dThdPase in various tumours and the adjacent non-neoplastic tissues. We assayed dThdPase activity by spectrophotometric means, and the expression of dThdPase was examined by immunoblotting and by immunohistochemical staining using a monoclonal antibody against dThdPase. In the oesophagus, stomach, colorectum, pancreas, and lung, dThdPase activity in carcinomas was significantly higher ($P < 0.05$) than that in the adjacent non-neoplastic tissues. The expression level of dThdPase detected by immunoblotting correlated well with the activity of dThdPase. In the oesophagus, stomach, colorectum, gall bladder, pancreas and lung, the proportion of dThdPase-positive tumours was significantly higher ($P < 0.05$ or 0.01) than that of the dThdPase-positive adjacent normal tissues. In oesophageal, gastric, colorectal and lung carcinomas, the proportion of dThdPase positivity in advanced carcinomas was significantly higher ($P < 0.01$) than that in early carcinomas. Tumour-infiltrative macrophages or lymphocytes in the lymph node, alveolar macrophages and Kupffer cells expressed high levels of dThdPase. The results indicate that dThdPase activity and expression level in many tumours are higher than those in the adjacent non-neoplastic tissues, and that dThdPase may have an important role in the proliferation of these solid tumours.

Key words: thymidine phosphorylase, platelet-derived endothelial cell growth factor, tumour infiltrative lymphocytes

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INTRODUCTION

THYMIDINE PHOSPHORYLASE (dThdPase; EC 2.4.2.4) catalyses the reversible phosphorolysis of thymidine, deoxyuridine and their analogues to their respective bases and 2-deoxyribose-1-phosphate [1–3]. It also catalyses the deoxyribosyltransfer from one deoxynucleoside to another base to form a second nucleoside [4–6]. The enzyme consists of two identical subunits, the molecular weight of each being about 55000 daltons in mammals [7–10]. It has been demonstrated that dThdPase is identical to platelet-derived endothelial cell growth factor (PD-ECGF) [11–14], and the enzymatic activity of dThdPase is needed for angiogenesis [15, 16].

Correspondence to S.-i. Akiyama.
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Plasma dThdPase activity has been shown to be elevated in cancer patients and tumour-bearing animals [17, 18], and its expression in some solid tumours is higher than that in the adjacent normal tissues [19–21]. The activity of dThdPase significantly correlates with the expression of thrombomodulin, a marker for endothelial cells in human colorectal carcinomas [20]. These reports suggested that dThdPase has an important role in angiogenesis in solid tumours. We prepared a monoclonal antibody against dThdPase and examined the level and distribution of dThdPase as well as dThdPase activity in various solid tumours.

MATERIALS AND METHODS

Preparation of monoclonal antibody against dThdPase

A GST fusion product containing 244 amino acid residues (amino acids 7–250) from the NH_2-terminus of PD-ECGF

was produced in bacteria and partially purified. A DNA fragment, containing roughly the 5′ half of the coding sequence of the PD-ECGF, was generated from the Xma digestion of plasmid pPL8 carrying the full length PD-ECGF cDNA kindly supplied by Drs K. Miyazono and C.H. Heldin (Ludwig Cancer Research Institute, Uppsala, Sweden). The resulting 0.8 kb fragment was purified and inserted into plasmid pGEX-2T (Pharmacia). After selecting transformed *E. coli* with ampicillin, a transformed colony was suspended in LB/ampicillin medium and vigorously agitated at 37°C until visibly turbid. The *E. coli* cells were further incubated with isopropyl-β-D-thiogalactopyranoside for 4 h at 37°C, pelleted and lysed with 2 × sample buffer (125 mM Tris–HCl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, and 0.002% bromophenol blue). The lysate was applied to preparative electrophoresis (Nihon Eido, NA-1800 type) and separated. Each 0.3 ml fraction was analysed by polyacrylamide gel electrophoresis (PAGE) and Coomassie blue staining. The five fractions that consisted mostly of a 53 kDa GST fusion product were collected (total protein was about 6.4 mg) and used to prepare a monoclonal antibody against dThdPase.

Specimens and tissue homogenate

Human carcinomas and adjacent non-neoplastic tissue specimens from different organs (5, oesophagus; 5, stomach; 5, colorectum; 5, liver; 5, pancreas; 3, common bile duct; 5, lung; 5, thyroid) and other normal tissues (small intestine, kidney, spleen, lymph node, muscle, placenta) obtained from surgery were frozen within 10 min at −80°C. Histopathological types of these carcinomas were squamous cell carcinoma (oesophagus), adenocarcinoma (stomach, colorectum, pancreas, common bile duct and lung), hepatocellular carcinoma, and papillary adenocarcinoma (thyroid). The tissues were homogenised in lysis buffer (50 mM Tris–HCl, pH 6.8, containing 1% Triton X-100, 2 mM PMSF and 0.02% 2-mercaptoethanol) and subjected to brief sonication. The lysates were centrifuged at 15000*g* for 30 min at 4°C. The supernatants were used for the enzyme assay and immunoblot analysis. The protein content was determined as described by Bradford [22].

Assay for dThdPase activity

dThdPase activity was assayed by the spectrophotometric procedure described by Friedkin and Roberts with some modifications [2]. Homogenised supernatants containing 400 μg protein were incubated with 0.1 M Tris–arsenate buffer (pH 6.5) containing 10 mM thymidine in a total volume of 0.1 ml. After incubation for 1 h at 37°C, the reactions were stopped by adding 1 ml 0.2 M NaOH, and the amount of thymidine formed was measured by absorbance at 300 nm. The dThdPase activity is expressed as the amount of thymine formed per mg protein per h.

Immunoblotting

Samples containing 50 μg protein were resolved by 11% SDS–PAGE, then proteins in the gel were electrophoretically transferred to a sheet of nitrocellulose [19]. After transfer, the nitrocellulose was treated with TTBS (400 mM NaCl, 20 mM Tris–HCl (pH 8.0), 0.05% Tween-20) containing 5.0% (w/v) skimmed milk for 1 h. The blots were incubated with the monoclonal antibody against dThdPase (1:1000 dilution) in TTBS containing 5% skimmed milk for 1 h. After washing four times in TTBS (10 min each), the nitrocellulose sheet was incubated with a peroxidase-conjugated horse anti-mouse IgG (Tago, Burlingame, California, U.S.A.) diluted 1:1000 in TTBS for 1 h. After washing in TTBS, the sheet was immersed in phosphate-buffered saline (PBS) containing 1 mg/ml of diaminobenzidine (DAB) and 0.03% (v/v) hydrogen peroxide. After development, the sheet was washed with distilled water and photographed.

Immunohistochemical detection and evaluation of dThdPase

Tumours and normal tissues resected from 31 patients with squamous cell carcinoma of the oesophagus, 28 with adenocarcinoma of the stomach, 39 with adenocarcinoma of the colon, 16 with hepatocellular carcinoma, 15 with adenocarcinoma of the pancreas, 16 with adenocarcinoma of the common bile duct, 10 with adenocarcinoma of the gall bladder, 30 with adenocarcinoma of the lung, and 20 with papillary adenocarcinoma of the thyroid, who underwent surgery in the First Department of Surgery, Kagoshima Unversity were included in this study. They did not receive irradiation and anticancer chemotherapy before surgery. All their tumours were completely resected at surgery and specimens immunostained using avidin-biotin-peroxidase complex (ABC) [23]. Samples were fixed with 10% formaldehyde in PBS, embedded in paraffin, and cut into 3-μ thick sections. The sections were deparaffinised with xylene and dehydrated with 98% ethanol. Endogenous peroxidase was blocked by immersing the slides in 0.3% hydrogen peroxide in absolute methanol for 30 min at room temperature. The sections were washed three times with PBS for 5 min, and incubated with 1% bovine serum albumin for 30 min at room temperature. The sections were then incubated at 4°C overnight with a monoclonal antibody against dThdPase or normal mouse immunoglobulin G_{2a} diluted 1000-fold with PBS containing 1% bovine serum albumin, then incubated for 30 min with biotinylated anti-mouse IgG at room temperature. After washing with PBS again, the sections were incubated with avidin and biotinylated horseradish peroxidase complex with PBS for 30 min. After washing in PBS, the immune complex was visualised by incubating the sections with 0.5 mg/ml DAB and 0.03% (v/v) H_2O_2 in PBS for 7 min. The sections were counterstained with haematoxylin and mounted.

Samples were considered to be dThdPase-positive when more than 5% of the parenchyma cells were stained, since less than 5% of cells were stained in most the normal tissues, except liver.

Statistical analysis

Statistical significance of the differences in dThdPase activity were examined using paired Wilcoxon rank-sum test. The result of immunohistochemical study was evaluated by the chi-square test. $P < 0.05$ was considered to indicate statistical significance.

RESULTS

Immunoblotting of dThdPase in tumours and the adjacent non-neoplastic tissues

We used a monoclonal antibody with which to examine the expression level of dThdPase in tissues. We detected dThdPase with a molecular weight of 55000 in several human normal tissues, carcinomas and the adjacent non-neoplastic tissues by immunoblotting. The expression level of dThdPase correlated with its activity. Among normal tissues, spleen, lung, liver and lymph node, had high levels of dThdPase

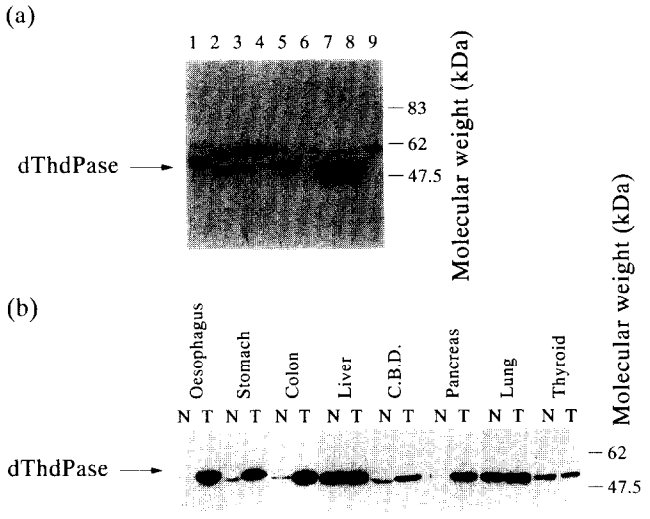

Figure 1. (a) The expression of dThdPase by immunoblotting with monoclonal antibody against dThdPase in human normal tissues. Lane 1, lung; 2, stomach; 3, colon; 4, small intestine; 5, liver; 6, kidney; 7, spleen; 8, lymph node; 9, muscle. (b) The expression of dThdPase by immunoblotting with monoclonal antibody against dThdPase in human carcinoma tissues and the adjacent non-neoplastic tissues. Representative cases are shown. N, normal; T, tumour; CBD, common bile ducts.

activity, whereas levels were low in the stomach, colon and small intestine (Figure 1a). The expression level of dThdPase detected by immunoblotting was higher in carcinomas of the oesophagus, stomach, colon, pancreas and lung than in the adjacent non-neoplastic tissues (Figure 1b). The expression level of dThdPase in normal liver was high and similar to that in hepatocellular carcinoma. The dThdPase expression was low in papillary adenocarcinoma of thyroid and similar to that in the adjacent non-neoplastic tissue.

The dThdPase activity in tumours and the adjacent non-neoplastic tissues

The dThdPase activity was significantly higher in carcinomas of oesophagus, stomach, colorectum, pancreas and lung than in the adjacent non-neoplastic tissue. In liver and thyroid, there was no significant difference between the dThdPase activity in carcinoma and that in the adjacent non-neoplastic tissue (Table 1). The dThdPase activity was correlated with the expression level of dThdPase determined by the immunoblot analysis in the tumours and the adjacent tissues (Figure 1 and Table 1).

Immunohistochemical detection of dThdPase

The dThdPase in normal tissues and the carcinomas with high dThdPase activity was localised by immunohistochemical staining using a monoclonal antibody against dThdPase (Figures 2 and 3). In the oesophagus, stomach, colon, gall bladder, pancreas and lung, the proportion of dThdPase-

Table 1. dThdPase activity in several human solid tumours and adjacent non-neoplastic tissues

Organ	Non-neoplastic tissue	Cancer tissue	Significance*
Oesophagus ($n = 5$)	5.43 ± 2.34	30.54 ± 4.28	$P < 0.05$
Stomach ($n = 5$)	5.83 ± 4.55	17.02 ± 5.32	$P < 0.05$
Colorectum ($n = 5$)	8.61 ± 5.20	17.40 ± 9.23	$P < 0.05$
Liver ($n = 5$)	22.05 ± 8.32	24.36 ± 7.90	NS
Pancreas ($n = 5$)	5.43 ± 1.23	18.98 ± 2.67	$P < 0.05$
Common bile duct ($n = 3$)	8.74 ± 3.43	9.78 ± 3.12	NS
Lung ($n = 5$)	14.24 ± 10.82	28.93 ± 11.19	$P < 0.05$
Thyroid ($n = 5$)	8.15 ± 4.86	9.34 ± 6.83	NS

*Significance was calculated by paired Wilcoxon test.
NS, not significant.
Mean ± standard deviation.

Table 2. The proportion of dThdPase positivity in several solid carcinomas and adjacent non-neoplastic tissues

Type of tissues	Number of dThdPase-positive/No. examined (%) Non-neoplastic tissue	Carcinoma	Significance
Oesophagus ($n = 31$)	2/31 (6.5)	20/31 (64.5)	$P < 0.01$
Stomach ($n = 28$)	2/28 (7.1)	16/28 (57.1)	$P < 0.01$
Colorectum ($n = 39$)	4/39 (10.3)	22/39 (56.4)	$P < 0.01$
Liver ($n = 16$)	16/16 (100)	16/10 (100)	NS
Gall bladder ($n = 10$)	1/10 (10.0)	7/10 (70.0)	$P < 0.05$
Bile duct ($n = 16$)	1/16 (6.3)	5/16 (31.3)	NS
Pancreas ($n = 15$)	2/15 (13.3)	9/15 (60.0)	$P < 0.05$
Lung ($n = 30$)	3/30 (10.0)	20/30 (66.7)	$P < 0.01$
Thyroid ($n = 20$)	1/20 (5.0)	4/20 (20.0)	NS

NS, not significant.

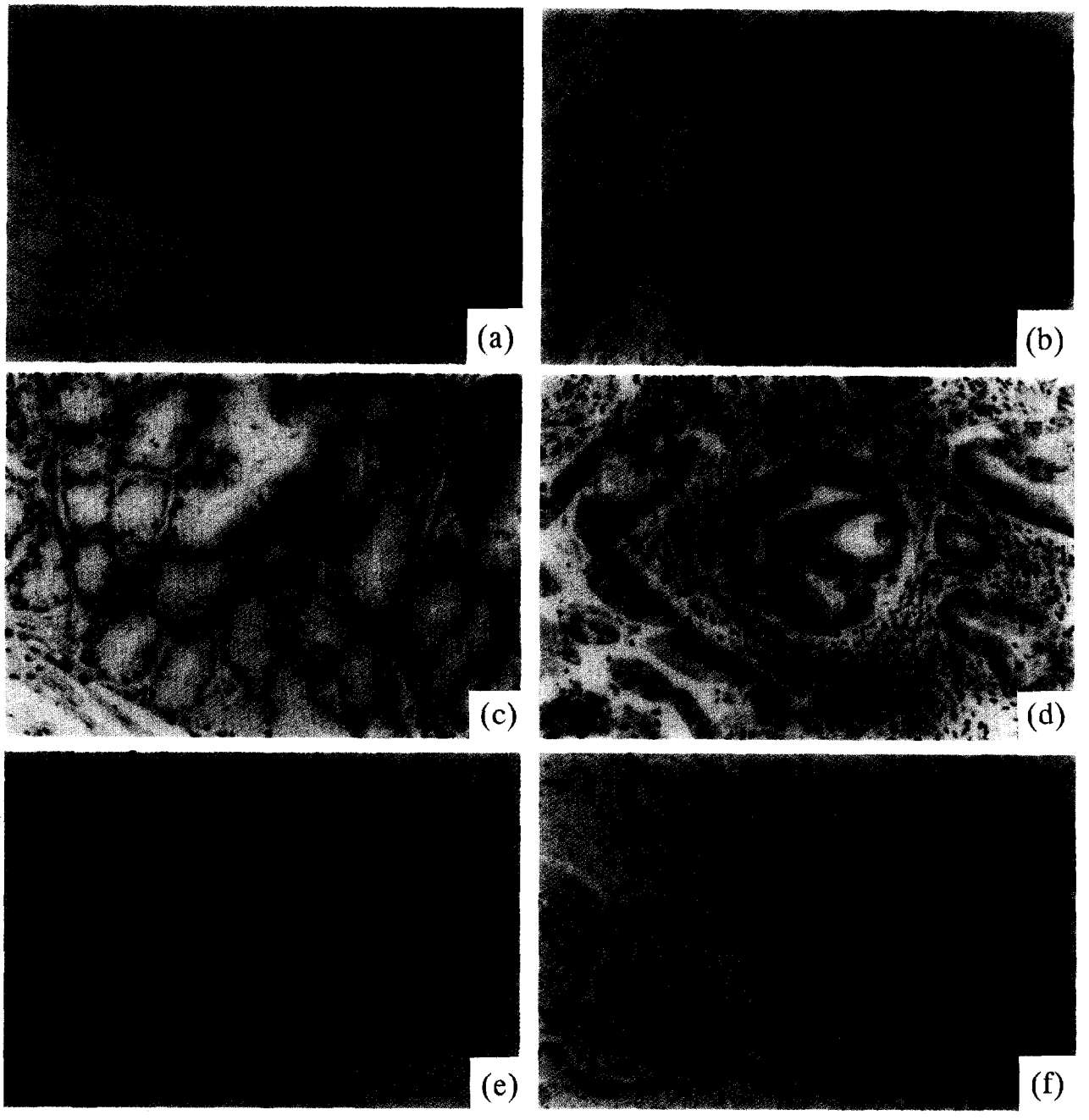

Figure 2. Immunohistochemical staining for dThdPase in normal oesophageal epithelium, × 200 (a), oesophageal carcinoma, × 400 (b), normal gastric mucosa, × 200 (c), gastric carcinoma, × 400 (d), normal colon epithelium, × 200 (e) and colon carcinoma, × 400 (f).

Table 3. The proportion of dThdPase positive tumours in early and advanced carcinomas

Type of tissues	Number of dThdPase-positive/No. examined (%) Early (stages I and II)	Advanced (stages III and IV)	Significance
Oesophagus ($n = 31$)	2/9 (22.2)	18/22 (81.8)	$P < 0.01$
Stomach ($n = 26$)	4/12 (33.3)	12/16 (75.0)	$P < 0.01$
Colorectum ($n = 39$)	4/14 (28.6)	18/25 (72.0)	$P < 0.01$
Lung ($n = 30$)	3/10 (30.0)	17/20 (85.0)	$P < 0.01$
Thyroid ($n = 20$)	1/8 (12.5)	3/12 (25.0)	NS

NS, not significant.

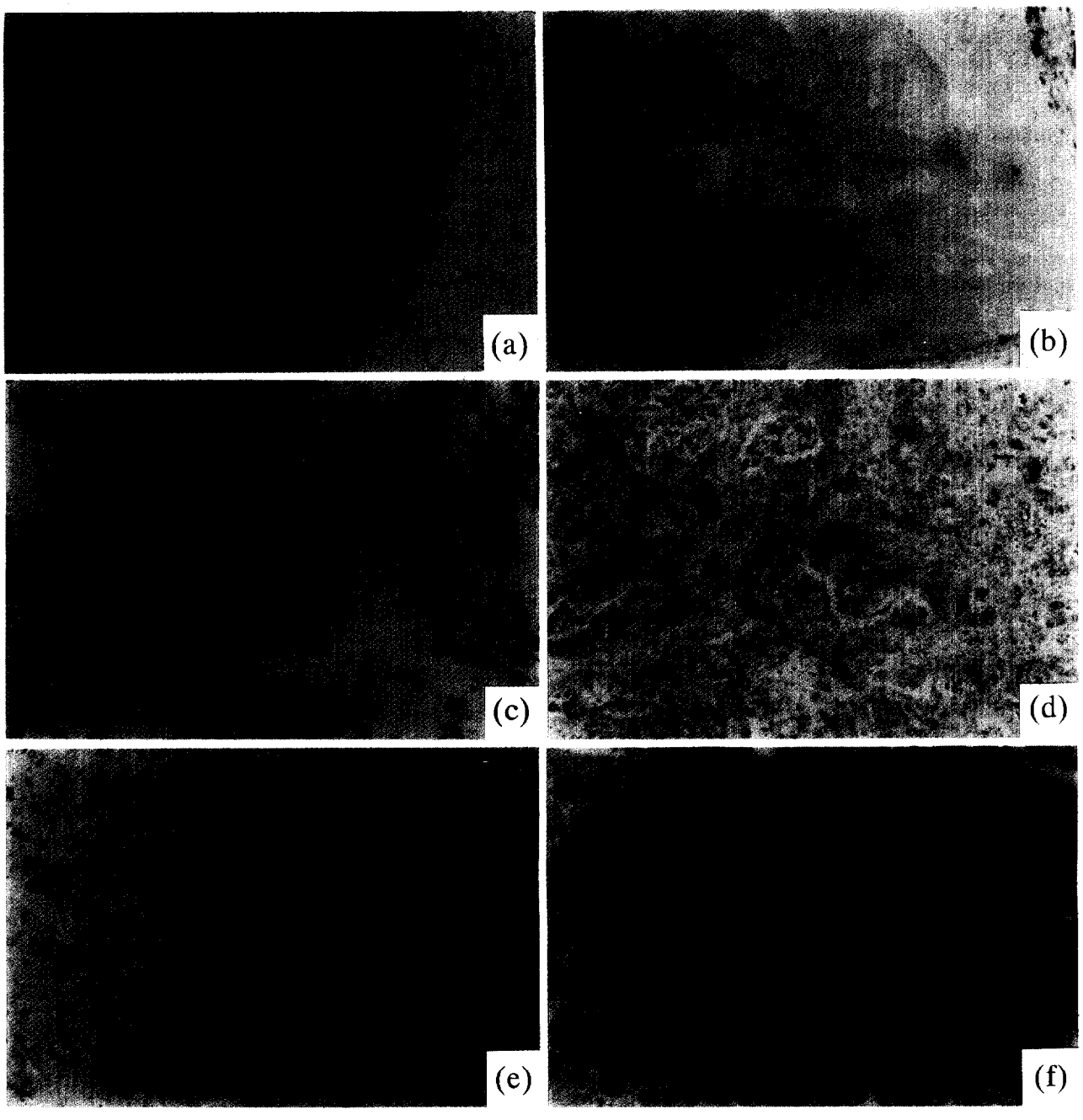

Figure 3. Immunohistochemical staining for dThdPase in normal lymph node, ×200 (a), metastatic lymph node from rectal carcinoma, ×200 (b), inflammatory lung, ×400 (c), lung carcinoma, ×200 (d), normal liver, ×200 (e) and hepatocellular carcinoma, ×400 (f).

positive tumours was significantly higher than that of dThdPase-positive adjacent normal tissues (Table 2). Squamous cell carcinomas, especially keratinised cells, were more intensely stained than adenocarcinomas (Figure 2b). Although all hepatocellular carcinomas (HCC) and normal liver tissues were dThdPase-positive, 2 of the 16 HCCs were more intensely stained than their adjacent normal tissues (Figure 3e,f). Extremely strong staining of Kupffer cells was observed in normal liver but not in HCC (Figure 3e,f). Normal lymphocytes in lymph nodes (Figure 3a), tumour infiltrative lymphocytes (Figure 3b), Kupffer cells (Figure 3e), alveolar macrophages (Figure 3c), and macrophages in several tissues also expressed high levels of dThdPase. Oesophageal, gastric, colorectal and lung carcinomas from patients in advanced stages (stages III and IV of TNM classification) [24] showed a higher proportion of dThdPase-positive tumours than those in early stages (stages I and II) (Table 3).

DISCUSSION

Our data and reports from other laboratories [11, 14, 25, 26] have suggested that human dThdPase is identical to PD-ECGF. dThdPase has angiogenic activity and its enzymatic activity is needed for angiogenesis [15, 16]. Tumour growth is dependent upon angiogensis [27], and the density of microvessels in histological sections significantly correlates with metastasis [28]. The expression of dThdPase has been shown to be increased in several types of malignant tumours [18–20]. We thus examined the expression of dThdPase in

various normal tissues and carcinomas by immunoblotting and by immunohistochemical staining using a newly prepared monoclonal antibody against dThdPase.

Distribution of dThdPase in normal tissues examined by this monoclonal antibody was almost the same as that shown in previous reports [19, 29]. In the immunohistochemical study, dThdPase was mainly localised in the cytoplasm and rarely in the nucleus. We have previously reported that perinuclear staining can be observed in paraffin-embedded sections, but not in frozen sections [19]. A nuclear localisation signal has not been found in the amino acid sequence of dThdPase [30]. However, further detailed examination is needed to elucidate the exact intracellular location of dThdPase since nuclear location of dThdPase has been reported [29].

Our results showed that the expression level and activity of dThdPase in the tested tissues were correlated and that the expression level of dThdPase in several tumours was higher than that in the adjacent non-neoplastic tissues. We examined the expression of dThdPase in many samples from various organs in this study, and extended the previous findings [19–21] to a broad range of tumours and normal tissues. We have also demonstrated that infiltrative macrophages or lymphocytes as well as carcinoma cells in various tumours expressed dThdPase. However, it is unclear whether the increase in dThdPase level in these infiltrative cells plays an important role in angiogenesis, the differentiation of the cells or the function of these differentiated cells. In the gastrointestinal tract and lung, carcinomas from the patients with advanced stages (stages III and IV) showed a higher proportion of dThdPase positivity than those with early stages (stages I and II). dThdPase may be involved in the progression of the tumours in these organs.

Since dThdPase activity and expression in many tumours from various organs are higher than those in the adjacent non-neoplastic tissues, and dThdPase has angiogenic activity [15, 16] and is able to promote tumour growth [31], dThdPase may have an important role in the proliferation of these solid tumours.

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