Preparation of Anti-human Thymidylate Phosphorylase Monoclonal Antibodies Useful for Detecting the Enzyme Levels in Tumor Tissues

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The antitumor activity of cytostatic 5'-deoxy-5-fluorouridine (5'-dFUr) depends on its being converted to 5-fluorouracil (5-FUra) by the enzyme thymidine phosphorylase (dThdpase, EC 2.4.2.4). We prepared mouse anti-human dThdpase monoclonal antibodies to serve as tools for clinical studies with this drug. Partially purified dThdpase obtained from HCT116 human colon cancer cells grown in athymic mice was used as an antigen for the immunization of mice. Six hybridomas were cloned which produced anti-human dThdpase antibodies, as detected by Western blot analysis with human dThdpase. With these antibodies, we developed an ELISA method sensitive enough to measure dThdpase levels, even in tumor tissue samples weighing as little as 10 mg. In addition, one monoclonal antibody was suitable for immunologically staining the enzyme in tumor tissues. Thus, these anti-human dThdpase monoclonal antibodies could be used to measure levels of the enzyme in tumor cells, which is essential for the activation of 5'-dFUr.

Key words thymidine phosphorylase; antibody; ELISA

Cytostatic 5'-deoxy-5-fluorouridine (doxifluoridine, 5'-dFUr) is being clinically used for the treatment of gastric, colorectal, breast, bladder and cervical cancers in Japan. It has been shown to be more effective than 5-fluorouracil (5-FUra), tegafur and 2'-dFUr (2'-deoxy-5-fluorouridine) in various mouse transplantable tumors. 1-3 We have shown that 5'-dFUr is a produg from which 5-FUra is generated by pyrimidine nucleoside phosphorylases (PyNPase), mainly uridine phosphorylase (UrdPase, EC 2.4.2.3) in mouse tumors and thymidine phosphorylase (dThdpase) in human tumors. 1,4,5 Human tumor cells, therefore, became much more susceptible to 5'-dFUr either in vitro or in vivo when they were transfected with the dThdpase gene, whereas the susceptibility to 5-FUra was increased only slightly. 6,7 In addition, we previously showed that tumor cells also became much more susceptible to 5'-dFUr when they had been previously exposed to the inflammatory cytokines tumor necrosis factor α (TNFα), interleukin-1β (IL-1β) and interferon γ (INFγ), which are able to up-regulate dThdpase gene expression. 8

In preclinical studies, 5'-dFUr appeared to show efficacy in tumors having certain levels of PyNPase. 9 Therefore, it is of interest to investigate whether PyNPase (dThdpase) could be a predictive factor for the efficacy of 5'-dFUr in patients. In the present study, we prepared anti-human dThdpase monoclonal antibodies (MoAbs) by using an enzyme purified from human colon cancer xenograft HCT116 in mice. With these antibodies, we developed methods for measuring enzyme levels and for staining the enzyme in tumor tissues. These methods with anti-human dThdpase MoAbs would be useful for analyzing and predicting the efficacy of 5'-dFUr.

MATERIALS AND METHODS

Animals and Tumor Male BALB/c nu/nu mice were obtained from Clea Japan Co., Ltd., Tokyo. The mice were observed for at least one week and then tested at the age of 5 to 6 weeks. Human colon cancer HCT116 cells were obtained from American Type Culture Collection, Maryland, U.S.A. A single cell suspension of tumor cells (5×10⁶) cultured with McCoy's 5A medium containing 10% fetal calf serum was inoculated subcutaneously into the mice. When the tumor size reached about 2 cm³, tumor tissues were excised for purification of dThdpase.

Purification of dThdpase Each HCT116 human colon cancer xenograft was homogenized with 4 volumes of a buffer containing 1 mM EDTA, 5 mM 2-mercaptoethanol, 100 μM (p-aminophenyl) methanesulfon fluoride (APMSF), and 50 mM potassium phosphate (pH 7.5). The homogenate was centrifuged at 100000×g for 1 h, at 4°C, and the protein fraction precipitating between 20 and 45% saturation with ammonium sulfate at 4°C was collected by centrifugation. The precipitate was dissolved in a buffer containing 1 mM EDTA, 5 mM 2-mercaptoethanol, 10 μM APMSF, and 20 mM potassium phosphate (pH 7.5), and then dialyzed against the same buffer. The dialyzed solution was then applied to a DEAE-Toyopearl column (25×2 cm: Tosoh) equilibrated with a buffer containing 1 mM EDTA, 5 mM 2-mercaptoethanol, and 20 mM potassium phosphate (pH 7.5, buffer A). dThdpase was eluted with a linear gradient of KCl (0—200 mM) in Buffer A, and the pooled active fractions were applied to a DEAE-5PW column (75×7.5 mm: Tosoh). dThdpase was eluted with a linear gradient of KCl (50—200 mM) in buffer A and re-chromatographed with the same DEAE-5PW column. The pooled and concentrated active fractions were further applied to a G3000SWXL column (300×7.8 mm: Tosoh), and dThdpase was eluted with buffer A. The active fractions were collected, and the dThdpase thus obtained was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) according to the method of Laemmli. 10 The protein concentration was determined by the method of Lowry et al. 11 using bovine serum albumin as a standard.

Immunization and Preparation of Hybridomas BALB/c mice were immunized with 50 μg of purified dThdpase in complete Freund's adjuvant. Boosters containing 50 μg of

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purified dThdPase in the incomplete Freund's adjuvant were then given two times at 3-week intervals. Three days after the final booster, the mice were killed and their dissociated spleen cells were fused with P3X63-Ag8.1U1 myeloma cells as described by Fazekas de St Groth and Scheidegger.12) Hybridoma clones were screened by Western blot analysis for those which produced anti-dThdPase antibody.

**Western Blot Analysis** Partially purified dThdPase fractions were electrophoresed on 5−20% SDS–polyacrylamide gradient gels (Page: A10o, Tokyo) according to the method of Laemmli,10) and proteins in the gels were electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA). The PVDF membrane was treated with a Tris buffered saline (20 mM Tris–HCl, 150 mM NaCl, pH 7.5) (TBS) containing 3% (w/v) skim milk for 1 h. Then, the membrane was incubated for 1 h at 37 °C with a culture supernatant of hybridomas to be tested for their productivity of dThdPase antibodies. The membrane was washed and then incubated with anti-mouse IgG + IgA + IgM-conjugated horseradish peroxidase (KPL, Goithursens, MD). After washing, the membrane was developed using the Konica Immunostain HRP-1000 (Konica, Tokyo).

ELISA A 96-well microtiter plate (Nunc-immunoplate Maxisorp, Nunc, Roskilde, Denmark) was incubated at 4 °C overnight with 10 μg/ml of the dThdPase MoAb 104B in 10 mM phosphate buffered saline solution (PBS, pH 7.6). The plate coated with the antibody was then incubated with 3% (w/v) skim milk in PBS (blocking buffer) for 1 h at room temperature. The plate was washed with PBS containing 0.05% Tween 20 and 0.05% sodium azide and kept at 4 °C until used. Test samples and standard solutions of dThdPase, which is the HCT116 tumor homogenate serially diluted with a blocking buffer, were dispensed onto the plate coated with the antibody. The plate was [1] incubated at 37 °C for 1 h and then washed with 0.05% Tween 20 in PBS (washing buffer); [2] incubated with dThdPase MoAb 232-2 at 1 μg/ml in blocking buffer for 1 h at 37 °C and washed; [3] incubated with 2000-fold diluted anti-mouse IgG(γ) conjugated with horseradish peroxidase (Bio-Rad, Hercules, CA) for 30 min at 37 °C and washed; and [4] incubated with a substrate solution containing 3,3′,5,5′-tetramethylbenzidine (TMB) and H₂O₂ (TMB microwell peroxidase substrate system, KPL) for 10 to 20 min at room temperature. The peroxidase reaction was stopped by the addition of 1 M phosphate solution, and the amount of dThdPase sandwiched with the two anti-dThdPase MoAbs was estimated by measuring its absorbency at 450 nm with a plate reader (Bio-Rad, model 3500). The amount of dThdPase was calibrated with that measured for the standard solutions.

**dThdPase Enzyme Assay** The enzyme activity was assayed by a method described by Eda et al.8) Briefly, tumor tissues were first homogenized in 10 mM Tris–HCl buffer (pH 7.4) containing 15 mM NaCl, 1.5 mM MgCl₂ and 50 mM potassium phosphate, and then centrifuged at 105000 × g for 90 min. The supernatant was dialyzed overnight against 20 mM potassium phosphate buffer (pH 7.4) and 1 mM 2-mercaptoethanol, and was then used as a source of crude dThdPase. The dThdPase reaction mixture (120 μl) contained 183 mM potassium phosphate (pH 7.4), 10 mM 5′-dFUrU, and the crude enzyme from human cells. The reaction was carried out at 37 °C for 60 min, and the amount of 5-FUra generated was measured after its separation from 5′-dFUrU in the HPLC column ERC-ODS-1171, with the solvent comprising 50 mM sodium phosphate buffer (pH 6.8) containing 5 mM 1-decane sulfonic acid: methanol = 85:15 (v/v).

**Reagents** 5-FUra was purchased from Kyowa Hakko (Tokyo, Japan). 1-(Tetrahydro-2-furanyl)-5-fluorouracil (Tegafur) was purchased from Taiho Pharm. Co. (Tokyo, Japan). 2′-dFUrU was obtained from Hoffmann-La Roche (Nutley, U.S.A.), and 5′-dFUrU was synthesized at Hoffmann-La Roche (Basel, Switzerland).

**Immunostaining of dThdPase in Tissues** Tissue samples were sectioned into 4 μm slices and placed on vectabond (Vector)-coated glass slides. The deparaffinized sections were placed in 0.1 M citrate buffer (pH 6.0) and heated in a microwave oven for 5 min twice (500 W). After being washed with PBS, the sections were treated with 3% (w/v) skim milk in PBS at room temperature and incubated with the primary antibody MoAb 654-1 (1 μg/ml) overnight at 4 °C. After another washing with PBS, the sections were further incubated with biotinylated anti-mouse IgG for 30 min at room temperature. Thereafter, the sections were treated with 1 mg/ml periodic acid in PBS for 10 min to block endogenous peroxidase activity. After being washed again with PBS, the sections were incubated with Avidin-Biotin Complex (Vectastain ABC kit: Vector, Burlingame, CA) for 30 min at room temperature, and developed with 1 mg/ml diaminobenzidine tetrahydrochloride in TBS containing 0.03% (v/v) hydrogen peroxide. The sections were also counterstained with methylgreen and mounted.

**Statistical Analysis** The statistic similarity of the correlation coefficient between dThdPase levels measured by the enzyme activity assay and ELISA was compared with the Fisher’s r to z transformation-test. The significance of differences between the dThdPase levels in cancer and normal tissues was analyzed using the Mann-Whitney U test. Similarities were considered to be significant when the probability (p) value was <0.05.

**RESULTS**

Purification of dThdPase Human dThdPase was purified 525-fold from HCT116 human colon cancer xenografts grown in athymic mice (Table 1). In a PAGE of the purified preparation, one main band (55 kDa) for dThdPase and a few minor bands were detected (Fig. 1). The purified enzyme preparation phosphorylated thymidine (370 μmol/mg/h), 2′-dFUrU (962 μmol/mg/h) and 5′-dFUrU (582 μmol/mg/h), but not uridine (18 μmol/mg/h). Tegafur, which is metabolized to 5-FUra by P-450,13) was also not well phosphorylated (10 μmol/mg/h). This substrate specificity pattern indicates that the purified preparation is indeed dThdPase.

Preparation of dThdPase MoAbs Mice were immunized with purified dThdPase for the preparation of MoAbs to this enzyme. Six hybridomas producing
anti-dThdPase MoAb, which was detected by Western blot analysis, were prepared from the spleen cells of the immunized mice. The following isotypes of these hybridomas were prepared: IgM, κ (104B) and IgG1, κ (232-2, 654-1, 322E, 49-4, and 204-1). In Western blot analyses of extracts of the HCT116 colon cancer xenograft and of human cancer tissues, these Ig fractions reacted only with protein which had a molecular mass of 55 kDa, which is identical to that of dThdPase (Fig. 2a–c).

**Application to ELISA Assay** A sandwich-type ELISA for human dThdPase was developed with the MoAbs 104B (IgM) and 232-2 (IgG1). Figure 3 compared the dThdPase levels in human cancer tissues measured by ELISA and by a conventional enzyme assay. The dThdPase levels measured by these assays correlated well with each other for breast, gastric and colorectal cancers. In addition, the dThdPase levels measured by either method were higher in cancer tissues than in normal tissues adjacent to the cancer tissues (Fig. 4). ELISA is more convenient than the enzyme assay and can be used to test pieces of human cancer tissues as small as about 10 mg.

**Immunostaining of dThdPase in Tumor Tissues** The dThdPase MoAbs were examined for their capability to stain dThdPase in human cancer tissues. Among the 3 MoAbs tested, only one antibody (654-1, IgG1) could

### Table 1. Purification of dThdPase from Human Colon Cancer Xenograft HCT116

<table>
<thead>
<tr>
<th>Purification</th>
<th>Activity (units)a</th>
<th>Total protein (mg)</th>
<th>Specific activity (units/mg protein)</th>
<th>Fold</th>
<th>Recovery (%)</th>
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<td>Crude extract</td>
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<td>13563</td>
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<td>100.0</td>
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<td>498.1</td>
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<td>G3000S.Wx1</td>
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<td>1.7</td>
<td>33486</td>
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<td>6.6</td>
</tr>
</tbody>
</table>

a) 1 unit of enzyme activity is defined as the quantity that catalyzes the formation of 1.0 μg 5-FUra per hour.

### Figures

**Fig. 1. SDS-PAGE Patterns of Active Fractions from Each Step of Purification**

1. crude extract of human colon cancer xenograft HCT116; 2. ammonium sulfate fraction; 3. DEAE-Toyopearl fraction; 4. DEAE-5PW fraction; 5. G3000S.Wx1 fraction; M, molecular weight markers. Arrow head (►) indicates bands corresponding to that of dThdPase (55 kDa).

**Fig. 2. Immunoblot Analysis of Human Cancer Tissue Extracts with Anti-dThdPase MoAbs**

Human cancer tissues were stained with a) MoAb 654-1 (1 μg/ml), b) MoAb 232-2 (100 μg/ml) and c) MoAb 104B (100 μg/ml). 1. human colorectal cancer; 2. human gastric cancer; 3. human breast cancer; 4. human colon cancer xenograft HCT116; M, molecular weight markers. Arrow head (►) indicates bands corresponding to those of dThdPase (55 kDa).
stain the enzyme in cancer tissues; the other antibodies reacted only weakly with the enzyme. In human breast cancer tissues, the MoAb 654-1 stained dThdPase, which was mainly located in the cytoplasm, in tumor cells. In contrast, the tumor stromal cells were only slightly stained (Fig. 5).

DISCUSSION

Previously, we showed that cytostatic 5'-dFUrd itself is not cytotoxic, but it exerts antitumor activity after its conversion to the active drug, 5'-FUra, mainly by dThdPase in humans and UrdPase in rodents.\(^1\)\(^4\)\(^5\) In preclinical studies, the antiproliferative activity of 5'-dFUrd depends on the presence of these enzymes in tumor cells,\(^9\) and human cancer cells transfected with the dThdPase gene became highly susceptible to 5'-dFUrd treatment both in vitro and in vivo.\(^6\)\(^7\) In clinical studies, however, the correlation between the efficacy of 5'-dFUrd and dThdPase activity in tumors has not yet been fully investigated, because assay methods for measuring small pieces of sample, such as biopsy samples, have not been available. In the present study, we purified human dThdPase, prepared MoAbs to this enzyme, and developed methods for detecting this enzyme by ELISA and for immunostaining tissue samples with the MoAbs. These methods would allow us to measure the enzyme levels in a small piece of sample tissue. In addition, the ELISA method is much more convenient as compared with the enzyme assay, where we measured the amount of the product 5'-FUra after its separation from the substrate 5'-dFUrd by HPLC.\(^8\)

Many methods for predicting the susceptibility of a cancer to various anticancer drugs before the start of treatment have been investigated. One approach has been to test the antiproliferative activities of drugs against tumor cells and tissues either in vitro or in vivo. Hormone receptor levels, such as those of the estrogen receptor, are being used as predictive factors for hormone therapy. For predicting the efficacy of drugs that are activated or inactivated in tumor tissues, the levels of the enzymes involved in the activation or inactivation in tumors could also be predictive factors. For 5'-dFUrd, dThdPase which generates 5'-FUra is essential for the drug's conversion to 5'-FUra in human cancer cells, and the ELISA and immunostaining methods developed in the present study should be useful for predicting 5'-dFUrd efficacy. Tumors with high levels of dThdPase would be more susceptible to 5'-dFUrd than to the parent drug, 5'-FUra, whereas those with low levels of this enzyme would be refractory to 5'-dFUrd treatment.

5'-dFUrd is converted to 5'-FUra by either dThdPase or UrdPase.\(^1\)\(^4\)\(^5\) Therefore, the sum of the levels of these two enzymes in tumors should be measured to most accurately predict susceptibility to 5'-dFUrd. However, the main enzyme which converts 5'-dFUrd to 5'-FUra in
humans is dThdPase. In the present study, we observed that dThdPase levels detected by ELISA and the measured enzyme activity involved in converting 5'-dFUrD to 5-FUra were well correlated. In addition, very little uridine was converted to uracil by extracts from various human cancer tissues, whereas both thymidine and 5'-dFUrD were well phosphorylated (data not shown). Therefore, only dThdPase would contribute to the prodrug activation of 5'-dFUrD to 5-FUra in humans, and the UrDase activity would be trivial.

Human dThdPase is reported to be identical to the platelet-derived endothelial cell growth factor, PD-ECGF, which is angiogenic in the chick embryo chorioallantoic membrane assay. This enzyme is preferentially located in various human tumors, such as breast, colorectal, gastric, and bladder, as compared with levels of the enzyme in their normal surrounding tissues. Recently, Toi et al. observed that dThdPase levels, detected with the anti-dThdPase MoAb 654-1, were associated with neovascularization in human breast cancer. O'Brien et al. also reported the association in bladder cancer. The methods developed in the present study for detecting dThdPase using MoAbs to this enzyme would be useful not only for predicting the clinical efficacy of 5'-FUrD, but also for understanding the roles of this enzyme in angiogenesis in tumor tissues.

REFERENCES