Thymidine Phosphorylase Expression Is Associated with Both Increase of Intratumoral Microvessels and Decrease of Apoptosis in Human Colorectal Carcinomas

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ABSTRACT

Thymidine phosphorylase (dThdPase) activity is associated with breast and urinary bladder carcinomas. These reports on human malignancies (9), including gastrointestinal (9–14), pancreatic (9, 15), breast (16–18), and urinary bladder (19) carcinomas. These reports have demonstrated the clinical and pathological significance of dThdPase expression. The results can be briefly summarized as follows: (a) immunohistochemistry shows positive dThdPase signals not only in cancer cells but also in normal mucosa as well as stromal macrophages, lymphocytes, and fibroblasts; and (b) the level of dThdPase expression is well correlated with the IMVD, tumor metastasis, and unfavorable prognosis of the cancer patients.

Recently, Takebayashi et al. (13) demonstrated that dThdPase expression is a prognostic factor independent of angiogenesis in human colorectal carcinomas. Similarly, Moghaddam et al. (18) reported an increase in growth of human breast carcinomas expressing dThdPase without an increase of IMVD. These results suggest that dThdPase might possess functions other than inducing intratumoral angiogenesis that affect tumor growth. This possibility prompted us to analyze the role of dThdPase expression in apoptotic cell death, tumor cell proliferation, and regulation of p53 expression using a large series of surgically removed specimens.

In this study, we focus our attention on colorectal carcinomas, about which few reports are available for naturally occurring or preoperative chemotherapyl-induced apoptosis (20–23). Apoptosis of colorectal carcinomas, however, has never been examined from the viewpoint of dThdPase expression. Here, we provide in vivo evidence that dThdPase expression is correlated with apoptosis of human colorectal carcinomas.

INTRODUCTION

dThdPase, 3 a member of the pyrimidine nucleoside phosphorylase family, is essential for DNA synthesis and limiting for cell growth. This enzyme also converts 5′-deoxy-5-fluorouridine to 5-fluouracil (1–4). It was recently shown that dThdPase is identical to platelet-derived endothelial cell growth factor, which has been shown to possess potent angiogenic activity.

dThdPase expression was noted in 13 of 36 (36.1%) Dukes’ A and B carcinomas and in 13 of 28 (46.3%) Dukes’ C and D carcinomas. At least 10 areas consisting of carcinoma cells with diffuse dThdPase expression from the 26 dThdPase-positive tumors (category I) and 10 areas without dThdPase expression from the 38 negative tumors (category II) were selected from each case. For stage A and B tumors, the mean IMVDs were 64.8 ± 33.7 in category I and 33.2 ± 12.6 in category II tumors, whereas for stage C and D tumors, the mean IMVDs were 77.6 ± 27.2 in the category I and 34.7 ± 14.0 in the category II tumors. The mean IMVD was significantly higher in category I than in category II tumors (P < 0.01). The mean apoptotic indices (AI; percentage of apoptotic cells) were 2.7 ± 1.7 in the category I and 5.4 ± 2.2 in the category II carcinomas of stages A and B and 1.4 ± 0.5 in category I and 5.3 ± 2.3 in category II carcinomas of stages C and D, and the value of the mean AI was significantly lower in category I than in category II (P < 0.01), regardless of the Dukes’ stage. AI and IMVD showed a significant inverse correlation (P < 0.001). There was no significant difference in the frequency of p53 expression between the two categories. These results indicated that dThdPase expression provides an advantage for tumor growth of human colonic carcinomas not only by increasing the intratumoral microvessels but also by attenuation of apoptosis, which might occur via a p53 gene-independent pathway.

MATERIALS AND METHODS

Tissue Samples. A series of 64 patients were preoperatively diagnosed with advanced colorectal carcinoma at Hiroshima Prefectural Hospital in 1997 and 1998. The patients, who underwent surgery, received neither chemotherapy nor radiation before the operation. Routinely processed, formalin-fixed, paraffin-embedded tissue blocks containing the principal infiltration of the tumoral mass were selected. The serial sections, 4 μm thick, were examined by light microscopy, immunohistochemistry, and the TUNEL procedure.

Immunohistochemistry. Dewaxed paraffin sections were immunostained by the streptavidin–biotin peroxidase complex method (24). Primary antibodies raised against Ki-67 (monoclonal antibody MIB-1, diluted 1:100; Immunotech, Marseille, France), CD34 (monoclonal antibody NU-4A1; Nichirei, Tokyo, Japan), p53 (monoclonal antibody BP53-12, diluted 1:50; Novocastra Laboratories Ltd., Newcastle, United Kingdom), vascular endothelial growth factor (polyclonal antibody A-20, diluted 1:50; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and dThdPase (mouse monoclonal antibody 654-1, diluted 1:1000; Nippon Roche Research Center, Kanagawa, Japan) were used. Trespin pre-treatment was performed for Ki-67 staining. All of the sections were heated in 10 mm citrate buffer (pH 6.0) in a microwave oven for 15 min at 94°C. Immunoreaction was visualized with 3,3′-diaminobenzidine, and the sections were counterstained with 3% methyl green.

TUNEL Method. To detect DNA breaks in situ, we performed TUNEL according to the method of Gavril et al. (25), using an Apop Tag Plus in situ apoptosis detection kit (Onchor, Gaithersburg, MD). Briefly, after deparaffinization and blocking of endogenous peroxidase with 2% hydrogen peroxide (H2O2) in methanol for 30 min at room temperature, sections were incubated with 20 μg/ml proteinase K (Boehringer Mannheim/Yamanouchi, Tokyo, Japan) for 40 min at 37°C. After prehybridization treatment, the sections were incubated with terminal deoxynucleotidyl transferase, digoxigenin-11-dUTP, and dATP in a moist chamber for 90 min at 37°C. Incubation

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3 The abbreviations used are: dThdPase, thymidine phosphorylase; IMVD, intratumoral microvessel density; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-digoxigenin nick end labeling; VEGF, vascular endothelial growth factor; AI, apoptotic index; KI, Ki-67 labeling index.

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with antidigoxigenin antibody-peroxidase for 30 min at room temperature was used for detection of digoxigenin-11-dUTP labeling, followed by color development with a solution containing 3,3′-diaminobenzidine and H₂O₂. Methyl green was used for counterstaining.

Assessment of p53 and dThdPase Immunostaining. We judged samples to be positive for p53 or dThdPase when at least 10% of cancer cells were stained.

Classification of the Cases and Determination of AI, KI, and IMVD. The AI (percentage of apoptotic cells) of each colorectal carcinoma was obtained as the number of TUNEL-positive cancer cells per 1000 cancer cells in the most frequently labeled area. The KI was calculated similarly. We evaluated mean AI, KI, and IMVD in areas that were selected using the following criteria: areas consisting of carcinoma cells with diffuse dThdPase expression from the dThdPase-positive tumors (category I) and areas without dThdPase expression from negative tumors (category II). For the determination of mean AI, KI, and IMVD in each case, we selected at least 10 areas in each category. The mean IMVD was determined by examining all of under a light microscope with a 200-fold magnification, as described by Weidner et al. (26). The average numbers were recorded as the IMVD for each case.

Statistical Analysis. Correlation between AI and IMVD was analyzed using the Spearman rank correlation coefficient. The Student’s t test was used for analyzing statistical correlations among IMVD, AI, KI, and p53 expression. P < 0.05 was considered significant.

RESULTS

dThdPase Immunoreactivity and IMVD. As described in previous reports (9, 10, 14, 27), the cytoplasmic and nuclear immunoreactivity was variably noted in colorectal carcinoma cells as well as in stromal lymphocytes, fibroblasts, and macrophages (Fig. 1, a and b). dThdPase immunoreactivity was observed in both the nucleus and cytoplasm of the colorectal carcinoma cells. The cytoplasmic immunoreactions were more predominant than the nuclear reactions. dThdPase expression was noted in 13 of the 36 (36.1%) Dukes’ A and B carcinomas and 13 of the 28 (46.3%) Dukes’ C and D carcinomas; the frequencies were not significantly different (Table 1). Thus, the dThdPase expression was not correlated with the tumor stage. We selected at least 10 areas containing cancer cells with diffuse dThdPase expression from the 26 dThdPase-positive cases (category I) and, similarly, at least 10 areas without dThdPase expression from the 38 negative cases (category II). Intratumoral microvessels were clearly demonstrated by CD34 immunostaining (Fig. 1c).

Table 1 shows the relationships among dThdPase expression, mean IMVDs, and the tumor progression of the colorectal carcinomas, according to the Dukes’ classification. For the stage A and B tumors, the mean IMVDs were 64.8 ± 33.7 in the 13 category I carcinomas and 33.2 ± 12.6 in the 23 category II carcinomas. Similarly, mean IMVD was 77.6 ± 27.2 in the 13 category I carcinomas and 34.7 ± 14.0 in the 15 category II carcinomas of the stage C and D. Mean IMVD was significantly higher in the dThdPase-positive category I than in the dThdPase-negative category II tumors (P < 0.01). The mean IMVD was not associated with the tumor stage in either category. Moreover, the frequency of VEGF expression tended to be higher in category I than it was in the category II, regardless of tumor stage. However, there was no significant difference.

Correlations between AI, KI, and IMVD and p53 Expression. Next, we analyzed the AIs and KIs in the colorectal carcinomas (Table 1 and Fig. 2, a and b). The mean AIs were 2.7 ± 1.7 in the 13 category I carcinomas and 5.4 ± 2.2 in the 23 category II carcinomas of Dukes’ A and B stages and 1.4 ± 0.5 in category I (n = 13) and 5.3 ± 2.3 in category II carcinomas (n = 15) of Dukes’ C and D stages. The mean AI was significantly lower in the dThdPase-positive carcinomas than in the negative carcinomas (P < 0.01), regardless of the tumor stage. On the other hand, the mean KI was significantly higher in the category I carcinomas than in the category II carcinomas of Dukes’ C and D (P < 0.01), whereas there was no significant difference in the mean KI between the two categories of the Dukes’ A and B stages.

Regression analysis of the Spearman rank correlation coefficient, on plots of AI versus IMVD on a per case basis, showed a significant inverse correlation between AI and IMVD (r = 0.51, P < 0.001; Fig. 3).

Nuclear p53 expression was noted in 12 (33.3%) of the Dukes’ A and B carcinomas and in 10 (35.7%) of the Dukes’ C and D carcinomas (Fig. 2c). Thus, the frequency of p53 expression, like the frequency of dThdPase expression, was not correlated with the tumor stage.

DISCUSSION

Angiogenesis is essential for tumor development and proliferation. Prevascular tumors might remain dormant in situ (<3 mm in diam-
the relationship between angiogenesis and tumor cell apoptosis. Lu

giogenesis and apoptosis. Little attention, however, has been paid to

impossible that there exists a close correlation between tumor an-

in vivo

tumor cells or normal cells
dormancy or dormancy-like behavior of colorectal carcinomas. They found the highest IMVD in the
colorectal carcinomas in adenomas, followed by adenomas with high
dysplasia and adenomas with low dysplasia; the differences among
these were significant. In the contrast, the AI was significantly higher
in the adenomas with low dysplasia than in the other two types of
lesions. These results, including those of the present study, might
suggest that apoptosis is directly enhanced by poorer blood supply in
colorectal adenomas and carcinomas.

On the other hand, Kitazono et al. (37) examined the relationship
between dThdPase expression and apoptosis using human cultured epidermoid carcinoma KB cells in vitro. They found that dThdPase as well
as a degradation product of thymidine, 2-deoxy-D-ribose, conferred re-
sistance to apoptosis induced by hypoxia in the KB cells, suggesting that
dThdPase has function other than stimulating angiogenesis that affect
tumor growth. dThdPase might be directly involved in intracellular ap-
optotic signal transduction, although the precise mechanism has not been
elucidated. This might explain why breast carcinoma cells that overex-
pressed dThdPase showed an increase in tumor growth without an in-
crease of IMVD (18) and also why dThdPase is a prognostic factor
independent of IMVD in colorectal carcinomas (13).

p53 protein has been shown to be required for the induction of the
apoptotic pathway triggered by oncogenic activation and expression of
cytotoxic genes (38, 39). The protein might sensitize damaged cells

ter) for months to years, followed by rapid growth, progression, and
even metastasis of a phenotypically transformed subclone of prevas-
cular cells possessing angiogenic activity (28, 29). Angiogenesis
inhibitors can induce and sustain dormancy of experimental primary
tumors and micrometastases by increasing tumor cell apoptosis, al-
though the proliferation rate remains unchanged (30–32). In this
study, significantly lower AI and higher IMVD were clearly demon-
strated in the dThdPase-positive colonic carcinomas compared with the
negative carcinomas, regardless of the tumor stage. This might
imply that dThdPase expression provides an advantage for tumor

growth of human colorectal carcinomas not only by forming a favor-
able, vascular-rich intratumoral microenvironment but also by allow-
ing cells to evade the apoptotic mechanism. Consequently, tumor
dormancy might easily be abolished, and tumor metastasis might
occur in the advanced colonic carcinomas. Moreover, KI was signif-
ically higher in the dThdPase-positive carcinomas than in the neg-
ative carcinomas of Dukes’ C and D, which showed metastasis to

development of cytotoxic genes (38, 39). The protein has been shown to be required for the induction of the
apoptotic pathway triggered by oncogenic activation and expression of
cytotoxic genes (38, 39). The protein might sensitize damaged cells

consistent with their report, in which the incidence of apoptosis was
significantly influenced by the extent of neovascularization, suggest-
ing that tumor angiogenesis might contribute to the reduction of
apoptosis in tumor cells. More recently, Aotake et al. (21) analyzed
the relationship between angiogenesis and apoptosis in human colo-
rectal adenomas and carcinomas. They found the highest IMVD in the
colorectal carcinomas in adenomas, followed by adenomas with high
dysplasia and adenomas with low dysplasia; the differences among
these were significant. In the contrast, the AI was significantly higher
in the adenomas with low dysplasia than in the other two types of
lesions. These results, including those of the present study, might
suggest that apoptosis is directly enhanced by poorer blood supply in
colorectal adenomas and carcinomas.

Table 1 Relationship between dThdPase expression and AI, KI, IMVD, p53, and vascular endothelial growth factor in human colorectal carcinomas

<table>
<thead>
<tr>
<th>Stage/category</th>
<th>IMVD</th>
<th>AI</th>
<th>KI</th>
<th>p53</th>
<th>VEGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dukes’ A and B</td>
<td></td>
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</tr>
<tr>
<td>Category I, dThdPase (+) (n = 13)</td>
<td>64.8 ± 33.7(\text{c})</td>
<td>2.7 ± 1.7(\text{c})</td>
<td>46.8 ± 18.4</td>
<td>6 (46%)</td>
<td>10 (77%)</td>
</tr>
<tr>
<td>Category II, dThdPase (-) (n = 23)</td>
<td>33.2 ± 12.6</td>
<td>5.4 ± 2.2</td>
<td>48.4 ± 16.3</td>
<td>6 (26%)</td>
<td>15 (65%)</td>
</tr>
<tr>
<td>Dukes’ C and D</td>
<td></td>
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<tr>
<td>Category I, dThdPase (+) (n = 13)</td>
<td>76.0 ± 27.2(\text{c})</td>
<td>1.4 ± 0.5(\text{c})</td>
<td>56.8 ± 14.0(\text{b})</td>
<td>5 (38%)</td>
<td>12 (92%)</td>
</tr>
<tr>
<td>Category II, dThdPase (-) (n = 15)</td>
<td>34.7 ± 14.0</td>
<td>5.3 ± 2.3</td>
<td>43.3 ± 15.2</td>
<td>5 (33%)</td>
<td>11 (73%)</td>
</tr>
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</table>

\(\text{c}\) Values are expressed as means ± SE or as n (%).
\(\text{b}\) VEGF, vascular endothelial growth factor.
\(\text{c}\) P < 0.01 vs. control.

Fig. 2. Serial sections from colonic carcinoma without dThdPase expression. a, a few apoptotic cells are noted (arrowheads). Apoptotic bodies showing fragmented nuclei are seen in the lumen of cancer glands. H&E staining. Magnification, ×200. b, TUNEL signals are observed in the nuclei of apoptotic cancer cells (arrowheads). TUNEL staining. Magnification, ×200. c, numerous cancer cells show nuclear immunoreactivity for p53. Magnification, ×200.
to apoptosis, acting to prevent the propagation of transforming mutations. Cells lacking a functional p53 protein would be refractory to this process of elimination and might proliferate more aggressively.

We have demonstrated that expression of a mutated p53 gene attenuates apoptotic cell death in human gastric carcinomas in vivo with marginal significance, implying that apoptosis occurs via both p53-dependent and -independent pathways (40). In this study, no apparent impact of aberrant p53 expression on angiogenesis and AI was noted. Thus, dThdPase-related attenuation of apoptosis is considered to occur in a p53 gene-independent pathway.

In summary, we have clearly demonstrated in vivo evidence that dThdPase expression attenuates apoptotic cell death and increases IMVD in human colorectal carcinomas, regardless of p53 expression.

The precise mechanism of the apoptosis resistance of dThdPase-expressing carcinoma cells awaits further clarification and might provide information valuable for angiogenesis-targeted therapy.

REFERENCES


Fig. 3. Spearman rank correlation coefficient test for the correlation between AI and IMVD (for all cases, r = 0.51, P < 0.001).

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