Significance of Thymidine Phosphorylase as a Marker of Protumor Monocytes in Breast Cancer

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ABSTRACT

Tumor-associated monocytic cells (TAMs) are a major component of the stroma responsible for tumor formation. TAMs generate various kinds of mediators for their function, one of which is thymidine phosphorylase (TP). TP is an angiogenic enzyme that is known to be up-regulated in tumor tissues. Here, we focused on the clinical implication of TP expression in TAMs by studying 229 primary breast carcinoma tissues.

Immunohistochemical analysis demonstrated that monocytic TP+ tumors had a significantly worse prognosis than did monocytic TP− tumors (P < 0.01, log-rank test). A multivariate analysis confirmed that monocytic TP status provided an independent prognostic value (P < 0.0001). Furthermore, of interest was that monocytic TP status could categorize the CD68+ patients, who had an extensive accumulation of CD68+ TAMs, into two subgroups with strikingly contrasting prognoses: a good prognostic monocytic TP− group and a poor prognostic monocytic TP+ group. This indicates that there are both antitumor and protumor types of TAM. Subanalysis showed that microvessel density was significantly increased in CD68+/monocytic TP+ tumors compared with CD68+/monocytic TP− tumors.

Experimentally, TAMs are known to function in diverse manners, antitumor and protumor; however, little is known about clinically recognizable markers to characterize the TAMs in histological sections. TP might be such a marker, which would be useful for identifying the character of TAMs, particularly the protumor phenotype.

INTRODUCTION

Solid tumors needs stromal formation for their growth. Tumor stroma includes vascular cells, fibroblasts, matrix components, and infiltrating blood cells. Tumor-infiltrating lymphocytes generally play a role in suppressing tumor spread, but TAMs do not necessarily show antitumor activity. TAMs can transmit signals to the immune network as antigen presentation cells and can directly attack tumor cells in vitro; however, TAMs located in situ can function as protumor stromal cells under a mechanism of paracrine control by the tumor cells. Tumor cells are known to produce a number of soluble mediators to recruit monocytes to the tumor tissue from the systemic circulation, including: C-C chemokines, such as monocyte chemoattractant protein-1; C-X-C chemokines, such as IL-8 and gro-α; and VEGF (2–4). Recruited TAMs can secrete protumor factors, including epidermal growth factor, VEGF, basic fibroblast growth factor, urokinase-type plasminogen activator, cathepsin D, and MMPs (1–8). In fact, TAM content was significantly associated with intratumoral urokinase-type plasminogen activator level, angiogenesis grade, and poor prognosis in primary breast carcinoma (9, 10). In mice, depletion of macrophages by whole-body X-irradiation inhibited the growth and neovascularization of fibrosarcomas (11). Pupa et al. (12) reported that poorly differentiated and c-erb B-2+ tumors were frequently infiltrated by TAMs, whereas the tumors with well-differentiated and c-erb B-2− phenotype were infiltrated by T lymphocytes instead. On the other hand, immune response through MHC class I molecules is reported to be generally suppressed in human breast carcinoma (13). These findings suggest that the function of TAMs is differentially augmented by tumor cells.

TP/platelet-derived endothelial cell growth factor is a unique enzyme that is involved not only in nucleoside metabolism but also in angiogenesis, where thymidine metabolites stimulate endothelial chemotaxis (14–17). TP expression is found to be markedly and selectively increased in tumor tissues compared with adjacent normal tissues in a variety of tumor types (18–20). However, only low levels or faint amounts of TP were detected in cultured human breast cancer cell lines, which suggests that TP expression in vivo is modulated by the tumor-stroma interaction (17). In this sense, several cytokines (tumor necrosis factor-α, IL-1, and IFN-γ) and hypoxia are known to be responsible for the up-regulation of TP (21, 22). According to immunohisto-

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2 The abbreviations used are: TAM, tumor-associated monocytic cell; IL, interleukin; VEGF, vascular endothelial growth factor; MMP, matrix metalloproteinase; TP, thymidine phosphorylase; MVD, microvessel density; ER, estrogen receptor; PR, progesterone receptor; 5FU, 5-fluorouracil; CI, confidence interval; TNF-α, tumor necrosis factor-α.
chemistry findings, TP is localized in various stromal cells, including fibroblastic, endothelial cells, and monocytic cells, as well as in tumor cells (23). Therefore, TP status seems to be a sensitive marker, which reflects whether or not these stromal cells are activated by the microenvironmental conditions formed by tumor cells. Here, we have examined TP expression in TAMs and assessed its biological and prognostic significance in primary breast carcinoma. These clinical data show that determination of TP is useful for identifying the character of TAMs, particularly the protumor forms.

PATIENTS AND METHODS

Two-hundred twenty-nine records of primary breast cancer patients who underwent mastectomy surgery from 1986 to 1996 in Tokyo Metropolitan Komagome Hospital were included in this study (average follow-up period, 48 months). The 3–5-μm sections of paraffin-embedded primary breast tumor tissues were analyzed by the indirect antiperoxidase immunocytochemical assay (Dako, Carpinteria, CA) for assessing the expression of TP, TAM count, and MVD. TAMs were stained by anti-CD68 monoclonal antibody (PG-M1; Dako A/S, Copenhagen, Denmark), and the positively stained cells were counted visually in the five most accumulated areas (“hot spots”) at the invasion front in the microscopic field (per mm²). The average count for the top three fields of five was calculated as the TAM count. TP staining was carried out with an anti-TP monoclonal antibody provided by Nippon Roche Research Center (Kamakura, Japan), and the TP+ monocytic cells were counted following the same procedure used for CD68+ cells. MVD was also determined by means of endothelial immunostaining with anti-factor VIII-related antigen monoclonal antibody (Dako, A/S, Denmark). Tumor cell TP status was also determined as tumor cell TP+ and tumor cell TP−, according to the staining intensity, as described previously (24). All immunohistochemical and pathological assessments were conducted by two pathologists under completely blind conditions with respect to clinical information. Hormone receptors, ER and PgR, were examined by enzymatic immunoassay in the cytoplasmic fraction of the tumor extract. Five fmol/mg protein was determined as the cutoff value for both ER and PgR.

The indication and schedule of adjuvant treatment were decided on the basis of patient characteristics, including axillary nodal involvement (N) and tumor size (T; based on the tumor-node-metastasis staging system), age, and ER status. Polychemotherapy, including cyclophosphamide, methotrexate, and 5FU (CMF), cyclophosphamide and 5FU (CF), and cyclophosphamide, epirubicin, and 5FU (CEF), was given for node-positive patients who were <55 years old, and tamoxifen was given for ER+ patients for at least 2 years. The patients were followed every 3 months, and recurrence was confirmed by histological examinations or objective image examinations. The relationship between the patients backgrounds and biological parameters was analyzed by the χ² test and unpaired Student’s t test. Survival curves were drawn by Kaplan and Meier method, and the difference in relapse-free survival was evaluated by log-rank test. A Beccel Mark-II analyzing system (Beccel, Tokyo, Japan) was used for multivariate analysis, which was assessed by Cox’s regression hazard model.

RESULTS

Stromal TP expression was detected in monocytic cells, fibroblasts, and endothelial cells, and the density of TP+ monocytic cells was counted. The TP+ monocytic cell count ranged between 0 and 505 counts/mm² (mean ± SD: 38.9 ± 72.6 counts/mm²). The CD68+ monocytic cell count varied from 5 to 517 counts/mm² (110.7 ± 95.3 counts/mm²; Fig. 1). Of 229 primary tumors, 98 (42.8%) showed more than 25 TP+ monocytic cells per mm² (monocytic TP+). In the background analysis, monocytic TP status was not significantly correlated with T, N, menopause, ER, or PgR; however, it was significantly
associated with angiogenesis. Monocytic TP count was significantly correlated with MVD (r = 0.330; z = 5.126; P < 0.001; 95% CI, 0.208–0.441; Fig. 1). The CD68+ cell count was also significantly correlated with MVD (r = 0.163; z = 2.467; P < 0.02; 95% CI, 0.34–0.287; Fig. 1), although the statistical correlation was less compared with the relationship between the monocytic TP count and MVD. Tumor cell TP expression was observed in 107 (46.7%) of 229 tumors; there was a significant correlation between tumor TP status and monocytic TP status (P < 0.01; Table 1).

Univariate prognosis analysis showed that monocytic TP status was a significant prognostic indicator. Monocytic TP+ tumors had a significantly worse prognosis compared with that for monocytic TP− tumors (log-rank test, χ² = 17.67; P < 0.01; Fig. 2). According to a 25-count step-wise assessment at 8 points from 25 to 200 counts/mm² to evaluate the cutoff value of monocytic TP status, the highest prognostic significance was observed at the point of 25 counts/mm². The prognostic analysis by the stepwise method failed to demonstrate any significant prognostic value for the of CD68+/monocytic TAM count (Fig. 2). Although tumor cell TP status had no significant prognostic value (Fig. 2), monocytic TP status was significantly prognostic in both tumor cell TP+ and tumor cell TP− patients as well as in both node-negative and node-positive patients (node-negative, P < 0.05; node-positive, P < 0.01; Fig. 2). Multivariate analysis with five variables, including nodal status, tumor size, ER, MVD, and monocytic TP status, all of which showed P < 0.2 in univariate analysis, demonstrated that the prognostic values of monocytic TP status, nodal status, MVD, and tumor size were independent (Table 2). In the combination analysis between CD68+ and monocytic TP status, monocytic TP status divided CD68+ patients, who had counts of more than 100 CD68+ cells per mm², into two subgroups: a good prognostic monocytic TP− group and a poor prognostic monocytic TP+ group (Fig. 2). In addition, other combination analysis showed that the phenotype of monocytic TP+ and high MVD had an extremely poor prognosis (Fig. 2).

Table 3 shows the relationship between CD68+/monocytic TP status and MVD. The average MVD count was markedly higher in CD68+/monocytic TP+ tumors than in CD68+/monocytic TP− tumors. The MVD of CD68+/monocytic TP− tumors was equivalent to that of CD68−/monocytic TP− tumors.

**DISCUSSION**

Here, we focused on the expression of TP in TAMs and found that monocytic TP expression was a potent prognostic indicator in primary breast carcinoma patients. In a multivariate analysis, its prognostic value was significantly as potent as nodal status and MVD. Furthermore, interestingly, the TP status categorized CD68+ tumors into two subgroups with strikingly
contrasting prognoses. The TP+ phenotype was a marker of poor prognosis, whereas a TP− phenotype indicated a good prognosis. Experimentally, the bifunctional activity of the monocytes has already been documented (1–3); however, as yet, little about them has been characterized in human tumors. We demonstrated here that TAMs consist of different subsets, from the standpoint of clinical outcome, and TP status can be a novel marker to discriminate the character of TAMs.

Although TP is one of the nucleoside metabolism enzymes, it is known to exert various biological effects on the growth of human carcinoma. In particular, TP is capable of stimulating new vessel formation because the metabolites of thymidine,

**Fig. 2** Prognostic value of monocytic TP status. a, tumor cell TP had no significant prognostic value. b, monocytic TP+ tumors had a significantly worse prognosis compared with monocytic TP− tumors (P < 0.01, log-rank test). c, CD68 count showed no significant prognostic value. d, combined analysis with overall monocytic accumulation assessed by CD68 count and monocytic TP status. Monocytic TP status divided the tumors with extensive accumulation of CD68+ TAMs (CD68+) into two subgroups: a good prognostic monocytic TP+ group and a poor prognostic monocytic TP+ group. e, nodal status and monocytic TP status. Monocytic TP status was a significant prognostic indicator in both node-negative and node-positive patients. f, the phenotype of monocytic TP+ and MVD+ having counts in excess of 100 provided an extremely poor prognosis.
especially 2-deoxyribose-1-phosphate, are chemotactic to the endothelium (16, 17). TP-overexpressing tumor cells grow faster and form more angiogenic tumors than do wild-type TP– tumor cells in nude mice (17). TP expression was positively associated with MVD and with poor prognosis in gastrointestinal cancer (18, 25, 26). In fact, monocytic TP status was associated with MVD and with poor prognosis in gastrointestinal cancers (18, 25, 26). Among the cytokines, TNF-α, IL-1, and IFN-γ are mainly derived from monocytic cells but IFN-γ is from neutrophils, natural killer cells, and T lymphocytes, which are obviously present to a lesser extent than TAMs in tumor tissues (1–4). The concentrations of TNF-α and IL-1 were differentially increased (27), but that of IFN-γ was extremely low in primary breast tumors. In this sense, we confirmed a significant correlation between intratumoral TNF-α and IL-1 concentrations and TP concentration. These two cytokines, TNF-α and IL-1, can stimulate the production of MMPs, particularly MMP-2 and MMP-9, in the stromal cells. In breast cancer tissues, MMP-2 is mainly derived from fibroblasts, and MMP-9 is from monocytic cells (28–31). Induced and activated MMPs accelerate the subsequent stromal reactions, including the release of matrix-anchored heparin-binding growth factors, which facilitate the following tissue degradation and tissue remodeling. TNF-α and IL-1 are also noted to induce the expression of endothelial adhesion molecules recognized by tumor cells. The orchestration between tumor cells and stromal cells through proteases might explain an aspect of the poor prognosis of monocytic TP+ tumors.

Likewise, hypoxia is a key player in the induction of stromal growth factors, including VEGF, basic fibroblast growth factor, and platelet-derived growth factor (32, 33). In the previous analysis, a coexpression of TP and VEGF was frequently detected in highly vascularized tumors, and those tumors showed a significantly poor prognosis (34, 35). Hypoxia and protumor cytokines seem to play important roles in the growth mechanism of monocytic TP+ tumors.

In contrast, CD68+ tumors with monocytic TP– phenotype had a good prognosis by the 2 × 2 factorial analysis, suggesting that the accumulation of TAMs is likely to function as suppressors of tumor growth. Recent studies raise an attractive hypothesis that TAMs are responsible for producing endogenous inhibitors, including angiotatin, because the elastase activity for the production of angiotatin from plasminogen was proven to be located in monocytic cells (36). This study showed that, among CD68+ tumors, monocytic TP– tumors had a markedly lower MVD as compared with monocytic TP+ tumors. Especially, in the CD68+ and monocytic TP– subgroup, a population with a very low level of MVD was observed, where TAMs might be releasing large amounts of inhibitors for the endothelium. It will be of interest next to identify the negative regulators in TAMs of human breast tumors.

In this study, we failed to confirm significant prognostic value of CD68 count (10). A direct explanation for the discrepancy between our study and that of Leek et al. (10) seems to be difficult; however, it might be possible to consider that a relatively larger number of monocytic TP+ cases were included or that the population of TP+ TAMs in a tumor tissue was higher in Leek’s study than in ours. Recently, Nagaoka et al. (37) also indicated the prognostic significance of TP expression in stromal cells.

From therapeutic points of view, several inhibitors of macrophage infiltration and TNF-α production are now under

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### Table 2 Multivariate analysis

<table>
<thead>
<tr>
<th>Variable</th>
<th>Coefficient</th>
<th>SE</th>
<th>(\chi^2) statistic</th>
<th>P</th>
<th>Risk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nodal status (−, +)</td>
<td>1.271</td>
<td>0.272</td>
<td>19.99</td>
<td>0.000007</td>
<td>6.49</td>
</tr>
<tr>
<td>Tumor size (&lt;3.0, ≥3.0 cm)</td>
<td>0.090</td>
<td>0.047</td>
<td>3.61</td>
<td>0.058</td>
<td>1.44</td>
</tr>
<tr>
<td>ER (−, +)</td>
<td>0.067</td>
<td>0.175</td>
<td>0.14</td>
<td>0.70</td>
<td>1.07</td>
</tr>
<tr>
<td>MVD (&lt;100, ≥100)</td>
<td>0.500</td>
<td>0.233</td>
<td>4.41</td>
<td>0.036</td>
<td>1.98</td>
</tr>
<tr>
<td>Monocytic TP (−25, ≥25)</td>
<td>0.977</td>
<td>0.278</td>
<td>18.34</td>
<td>0.000018</td>
<td>4.03</td>
</tr>
</tbody>
</table>

### Table 3 CD68/Monocytic TP status and microvessel density

<table>
<thead>
<tr>
<th>Case no.</th>
<th>CD 68 status</th>
<th>Monocytic TP status</th>
<th>MVD* (counts/mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>61</td>
<td>+</td>
<td>+</td>
<td>120.7 ± 53.3</td>
</tr>
<tr>
<td>40</td>
<td>+</td>
<td>−</td>
<td>69.8 ± 31.5</td>
</tr>
<tr>
<td>37</td>
<td>−</td>
<td>+</td>
<td>104.0 ± 45.1</td>
</tr>
<tr>
<td>91</td>
<td>−</td>
<td>−</td>
<td>71.6 ± 36.2</td>
</tr>
</tbody>
</table>

* Values are means ± SD.

3 Unpublished data.
clinical investigation (38). TP-dependent 5FU prodrugs show antitumor activity for various types of human tumors (39, 40). In a recent cohort study, TP status was also characterized as a predictive marker of chemotherapy in primary breast cancer (41). The biological and clinical implications of TP, particularly monocytic TP status, should be extensively studied in other types of solid tumors.

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