Pigment Epithelium-derived Factor as a New Diagnostic Marker for Melanocytic Tumors

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Summary: Pigment epithelium-derived factor (PEDF), a potent antiangiogenic factor, has been lately known to correlate well with angiogenic and metastatic potentials of tumor cells. We investigated the expression of PEDF protein in various types of human tumor cells by an immunohistochemical technique using a monoclonal antibody. Consequently, we found the significantly frequent and intense expression of PEDF in human melanocytic tumor cells including malignant melanoma as compared to non-melanocytic ones. We evaluated the diagnostic usefulness of anti-PEDF antibody in melanocytic tumors by comparing its immunoreactive sensitivity with that of other conventional melanocytic markers such as S-100 protein, HMB-45 and Melan-A, and found that PEDF possess the equal ability to others on its sensitivity. We finally concluded that PEDF is a useful diagnostic marker for melanocytic tumors, especially malignant melanomas, by its use in combination with other markers.

Key words: pigment epithelium-derived factor (PEDF), malignant melanoma, melanocytic nevus, immunohistochemistry and angiogenesis

INTRODUCTION

Angiogenesis, regulated by a net balance between positive and negative factors of neovascularization produced by tumor and other cells, is essential for the sustained growth, invasion and metastasis of solid malignant tumors [1-3]. Tumor cells secrete various molecules inducing angiogenesis such as vascular endothelial growth factor and fibroblast growth factor, which promote endothelial cell proliferation, migration and eventually capillary tube formation [4-9]. In contrast, angiogenic inhibitors, including angiotatin, thrombospondin, and endostatin are also produced by tumor cells [10-12].

Pigment epithelium-derived factor (PEDF), a Mr 50,000 secreted glycoprotein, was first identified in conditioned medium of cultured fetal retinal pigment epithelial cells [13], and has been shown to exist in fetal and adult liver, testis, ovaries, placenta, brain, pancreas and prostate [14,15]. PEDF resembles, in sequence and structure, members of the serine protease inhibitor (serpin) family but lacks protease inhibitor activity itself [16]. Recently, PEDF was implicated in inhibition of angiogenesis in a dose-dependent manner both in vitro and in vivo [16-18]. PEDF inhibits endothelial cell migration and proliferation and has been shown to inhibit choroidal and retinal neovascularization [19-22]. The antiangiogenic efficiency of PEDF is more potent than that of other endogeneous angiogenic inhibitors including angiotatin, thrombospondin-1 and endostatin [23]. Interestingly, several reports demonstrated the

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Abbreviation: PEDF, pigment epithelium-derived factor.
expression of PEDF in various types of tumor cells such as neuroblastoma, glioma, prostatic carcinoma, pancreatic adenocarcinoma, oral squamous cell carcinoma and melanoma cells, in which PEDF expression was correlated well with angiogenic and metastatic potentials of tumor cells and patient's prognosis [15,24-29]. Furthermore, more recent reports showed that tumor growth and/or invasion was significantly inhibited by PEDF gene transfer into tumor cells [30-34].

In this paper, we analyzed the expression of PEDF in various types of human tumor cells by immunohistochemistry using a monoclonal antibody specific to human PEDF protein. Surprisingly, we found the significantly high and frequent expression of PEDF protein in human melanocytic tumor cells as compared with non-melanocytic ones. Then we sought to determine the usefulness of PEDF as a diagnostic marker for melanocytic tumors by comparing its immunoreactivity with that of other conventional melanocytic markers such as S-100 protein, HMB-45 and Melan-A. Finally we concluded that PEDF could be a useful diagnostic marker for melanocytic tumor, especially malignant melanomas because of its similar sensitivity to other established markers.

MATERIALS AND METHODS

Tumor tissue for immunohistochemistry

We retrieved 87 formalin-fixed, paraffin-embedded tissue blocks from 33 patients with malignant melanoma (23 primary and 10 metastatic melanoma), 20 patients with melanocytic nevus (14 intradermal nevus and 6 compound nevus) and 34 patients with non-melanocytic neoplasms (24 carcinomas, 2 glioblastomas, 4 neuroblastosomas, 1 paraganglioma and 2 retinoblastomas) from the files of the Department of Pathology at the Kurume University. Among the melanomas, 22 cases were from cutis or subcutis, 6 from nasal cavity, 4 from lymph node and one from rectum. All subcutaneous and lymph nodal lesions were metastatic cases, while others were primary cases. The primary sites of carcinomas were as follows: 6 stomach, 6 colon and rectum, 5 lung, 4 breast, one pancreas, one bile duct and one liver.

Immunohistochemical method for tumor tissues

Immunohistochemical studies were performed using standard protocols. The following primary antibodies were used: a monoclonal antibody specific to human PEDF (1/200 dilution; Corporation, Temecula, CA, USA), a polyclonal antibody to S-100 protein (1:1000 dilution; Dako Corporation, Carpenteria, CA, USA), a monoclonal antibody to HMB-45 antigen (1/50 dilution, Dako Corporation) and a monoclonal antibody to Melan-A (1/25 dilution, Novocastra Lab. Ltd., UK). Immunoperoxidase staining for formalin-fixed and paraffin wax-embedded tissue sections was performed using an ordinary biotin-streptavidin method. Briefly, 5-μm sections placed on lysine-coated glass slides were deparaffinized and rehydrated in a descending alcohol series. For staining for PEDF and Melan-A, slides were autoclaved in 10 mM citrate buffer (pH 6.0) at 121 °C for 15 min prior to blocking endogenous peroxidase activity and non-specific binding sites. For S-100 and HMB-45 immunostaining, the tissues on slide glasses were pretreated with trypsin prior to blocking. The sections were then incubated with primary antibodies for 1 hr at room temperature. In subsequent steps, we used the Dako LSAB2 kit/HRP (Dako) using 3-amino-9-ethyl carbazole (AEC) as a chromogen. The sections were then counterstained with hematoxylin. We also performed additional staining without primary antibody in parallel as a negative control to confirm specific immunoreactivity.

Evaluation of immunohistochemical staining

Visual observation scored each section on a scale from 0 to 3+ by positive percentage of tumor cells. To estimate the percentage of stained cells, the numbers of positive and negative tumor cells in the field were counted by three independent pathologists, and the number of positive cells to the total number of tumor cells was expressed as the percentage. The mean percentage was used for the scoring. The highest staining, in which more than 50% tumor cells were positive for staining, was scored as 3+. The lowest was scored as 1+ when only less than 20% of tumor cells displayed positive staining, and no staining at all was scored as 0. Positive tumor cells more than 20% and less than 50% were scored as 2+. For the comparison of immunoreactivity between PEDF and each other melanocytic marker, a case scored as other than 0 was classified into positive (+).

Statistical analysis

The correlations of immunoreactive scores for PEDF protein between melanocytic and non-melanocytic tumors, or between malignant
melanoma and melanocytic nevus, were analyzed by Mann-Whitney’s U test. The immunoreactive sensitivity between each antibody in melanocytic tumors was analyzed by Fisher’s exact probability test.

RESULTS

Frequent and intense PEDF protein expression in melanocytic tumor cells

Recent reports demonstrated the expression of PEDF in various types of tumor cells [15,24-29], many of which suggested the antiangiogenic effect of PEDF protein. Therefore, we first sought to investigate whether PEDF expression was specific for some kinds of tumor cells. First of all, we tested a various type of carcinoma cells for PEDF expression by an immunohistochemical technique. As shown in Table 1, most (approximate 80%) carcinoma cells did not express PEDF at all, although each one case of gastric, colonic and pulmonary adenocarcinoma, ductal carcinoma of the breast and tubular adenocarcinoma of the pancreas showed a small amount of positive cells (less than 5% of total tumor cells). PEDF protein was localized in the cytoplasm and showed a granular pattern in all these carcinoma cases (data not shown). As several non-epithelial benign or malignant tumor cells were also shown to express PEDF [24,25,29], we also screened the expression of PEDF protein in non-epithelial neoplasms. As shown in Table 1, we were not able to recognize PEDF protein expression in non-melanocytic tumors such as glioblastoma, ganglioneuroma, neuroblastoma, paraganglioma and retinoblastoma, although some of which were reported to express PEDF protein in tumor cells [24,25]. Surprisingly, we found frequent and intense PEDF expression in several cases of malignant melanoma cells (Fig. 1), which drove us to analyze much more cases of malignant melanoma as well as melanocytic nevus cells as a benign melanocytic tumor counterpart. PEDF expression in melanoma cells was specific, because we did not see any signals when we used mouse serum instead of anti-PEDF antibody (Fig. 1D). We eventually tested total 33 cases of malignant melanoma (cutaneous 19, sinonasal 6, rectal 1, lymph nodal 4) and 20 cases of melanocytic nevus. Results as shown in Table 1 demonstrated that 31 cases (94%) express PEDF protein among 33 cases of malignant melanoma, while positive cases are 15 (75%) in melanocytic nevus. On the other hand, only 5 non-melanocytic

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>Immunoreactivity with PEDF antibody</th>
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<tr>
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<td>-</td>
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<tr>
<td>Non-melanocytic tumor</td>
<td></td>
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<tr>
<td>Carcinoma</td>
<td></td>
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<tr>
<td>Stomach (n=6)</td>
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<tr>
<td>Colon and rectum (n=6)</td>
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</tr>
<tr>
<td>Lung (n=5)</td>
<td>4</td>
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<tr>
<td>Breast (n=4)</td>
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<tr>
<td>Pancreas (n=1)</td>
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</tr>
<tr>
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<tr>
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<tr>
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<tr>
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<tr>
<td>Paraganglioma (n=1)</td>
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<tr>
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<tr>
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<tr>
<td>Melanocytic nevus (n=20)</td>
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<tr>
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</tr>
<tr>
<td>Primary (n=23)</td>
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<tr>
<td>Metastatic (n=10)</td>
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Date are given as number (percentage). *: p<0.01 (Mann-Whitney’s U test)

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tumors (15%) expressed this protein. Statistical analysis demonstrated the significant difference between melanocytic and non-melanocytic tumors (p<0.01), but not between malignant melanoma and melanocytic nevus (p=0.093). These results indicated that melanocytic tumor cells express PEDF protein without distinction of biological behavior. Although we also compared the expression level of PEDF protein between 23 primary and 10 metastatic melanomas, we could not see any significant differences between them (p=0.287).

**Anti-PEDF antibody as a diagnostic marker for melanocytic tumors**

As we can see the frequent and intense expression of PEDF in melanocytic tumors, we next sought to examine whether we can use anti-PEDF antibody as a diagnostic marker. For this purpose, we compared the PEDF immunoreactive sensitivity for melanocytic tumor cells with other conventional melanocytic markers including S-100, HMB-45 and Melan-A, which are well known to be useful melanocytic tumor markers. Results as shown in Table 2, demonstrated that immunoreactive sensitivity between each melanocytic marker including PEDF was quite similar in malignant melanoma, although the sensitivity of PEDF was slightly less as compared with that of S-100 and Melan-A in melanocytic nevus. The expression intensity and frequency of each melanocytic marker was different.

**TABLE 2.**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Nevis</th>
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<th>Melanoma</th>
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<tbody>
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<td></td>
<td>-</td>
<td>+</td>
<td>(%)</td>
<td>-</td>
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<tr>
<td>PEDF</td>
<td>5</td>
<td>15</td>
<td>75</td>
<td>2</td>
</tr>
<tr>
<td>S-100</td>
<td>0</td>
<td>20</td>
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<td>HMB-45</td>
<td>13</td>
<td>7</td>
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</tr>
<tr>
<td>Melan-A</td>
<td>0</td>
<td>20</td>
<td>100</td>
<td>2</td>
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</table>

*:* p<0.05, *:** p<0.01 (Fisher's exact probability test)

*Fig. 1.* Histopathological and immunohistochemical studies of PEDF protein in malignant melanoma cells.

A and B: H&E staining of a section that contained epithelioid malignant melanoma cells. Low (A) and high (B) magnification. C: A section of the same case as A and B stained by monoclonal anti-PEDF antibody showing cytoplasmic localization of PEDF protein in malignant melanoma cells. D: Negative control staining without primary antibody with the same technique as demonstrated in C. NMS: normal mouse serum.
Fig. 2. Immunohistochemical study of PEDF and other melanocytic markers in malignant melanoma cells.
Sections from two different cases (case 1: A-D, case 2: E-H) of malignant melanoma were immunohistochemically stained with anti-PEDF (A, E), anti-S-100 (B, F), anti-HMB-45 (C, G) and anti-Melan A (D, H) antibodies as described in Materials and Methods. Case 1 exhibited diffuse and strong expression of PEDF and Melan A, but focal and weak expression of S-100 and HMB-45, while diffuse and strong expression of PEDF, HMB-45 and Melan A, but focal S-100 expression in case 2.
depending on the case. For example, two malignant melanoma cases as presented in Fig. 2, demonstrated very weak and focal expression in S-100 and HMB-45 proteins (Figs 2B and C) and in S-100 protein (Fig. 2F), respectively. We finally concluded from these results that we can use an anti-PEDF antibody as a melanocytic tumor marker in combination with other markers, especially in malignant melanoma.

DISCUSSION

PEDF, a potent antiangiogenic factor, has been lately known to correlate well with angiogenic and metastatic potentials of tumor cells. Therefore, we originally sought to screen various types of human tumors for the PEDF protein expression in relation to malignant behavior and prognosis, although the expression of this protein in tumor cells was already demonstrated in sporadic reports [15,24-29]. This is the first report in which many types of tumor cells were screened for the PEDF expression as we know, and we clearly demonstrated extremely frequent and intense PEDF expression in melanocytic tumor cells including malignant melanoma using paraffin blocks prepared from patients. A study on antiangiogenic effects of PEDF, in which cultured malignant melanoma cells as well as human melanocytes express PEDF protein, was recently reported [29]. In this report, they demonstrated the overexpression of PEDF suppressed angiogenesis and growth of melanoma cells.

Malignant melanoma is one of the highly aggressive tumors associated with high mortality rate because of frequent metastatic dissemination potential and acquisition of chemoresistance [35-37]. We originally expected lower or less expression level of PEDF protein in melanoma cells because of their highly metastatic potential. Unexpectedly, melanoma cells showed much higher expression of PEDF protein as compared with non-melanocytic malignant tumors including carcinomas, which indicated that highly aggressive potential in melanoma cells might be caused by several other factors than angiogenesis. Supporting this hypothesis, we also demonstrated frequent PEDF expression in melanocytic nevi, which are benign melanocytic tumors and do not exhibit invasive and metastatic activities. Thus, our results in this report suggested that PEDF could not be a prospective maker for tumor growth and metastasis. Further detail studies on the relationship between PEDF expression in tumor cells and their growth and invasiveness, are required.

In order to examine the usefulness of PEDF antibody for the pathological diagnosis of melanocytic tumors, we compared PEDF immunoreactivity in melanocytic tumors with routinely-used other markers such as S-100, HMB-45 and Melan-A. We found the similar sensitivity on PEDF expression to other conventional markers in melanocytic neoplasms, especially malignant melanoma. As non-melanocytic tumors did not show significantly frequent and strong PEDF expression, the specificity in this marker also appears to be enough for the diagnosis, although we need to confirm this to analyze more cases. We eventually concluded that we could use an anti-PEDF antibody for the diagnosis of melanocytic tumors in combination with other established markers.

REFERENCES


