Tumor Cell Expression of Programmed Cell Death-1 Ligand 1 Is a Prognostic Factor for Malignant Melanoma

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BACKGROUND: Melanoma tends to be refractory to various immunotherapies because of tumor-induced immunosuppression. To investigate the mechanism underlying the immunosuppression of melanoma patients, the authors focused on programmed cell death-1 (PD-1)/PD-1 ligand 1 (PD-L1) interaction between tumor cells and T cells.

METHODS: Melanoma specimens were collected from 59 primary tumors, 16 lymph nodes, and 4 lesions of in-transit metastasis. Specimens stained with anti-PD-L1 monoclonal antibodies were digitalized to jpg files. To evaluate the intensity of PD-L1 expression, histograms were used, and the red density (RD) was measured. PD-1 expression on T cells was analyzed in blood samples from 10 patients who had stage IV melanoma and in 4 samples of in-transit metastases.

RESULTS: Twenty-five patients comprised the “low” PD-L1 expression group (RD value, <90), and 34 patients comprised the “high” group (RD value, ≥90). Breslow tumor thickness in the high-expression group was significantly higher than in the low-expression group. Univariate and multivariate analyses revealed that the overall survival rate of the high-expression group was significantly lower than that of the low-expression group. In all patients with stage IV disease who were examined, both CD8-positive and CD4-positive T cells had significantly higher PD-1 expression levels in the peripheral blood. Tumor-infiltrating T cells expressed high levels of PD-1, and its expression was elevated further during the clinical course.

CONCLUSIONS: The current results indicated that there is a correlation between the degree of PD-L1 expression and the vertical growth of primary tumors in melanoma. Multivariate analysis demonstrated that PD-L1 expression is an independent prognostic factor for melanoma. Cancer 2010;116:1757–66. © 2010 American Cancer Society.

KEYWORDS: melanoma, peripheral blood mononuclear cells, programmed cell death, tumor-infiltrating lymphocytes.

Although malignant melanoma is a representative immunogenic tumor among various neoplasms,1 it tends to be refractory to immunotherapy.2,3 The presence or absence of tumor-infiltrating lymphocytes is 1 of several hallmarks that predict prognosis for patients with melanoma.4 High frequencies of tumor-infiltrating, CD8-positive lymphocytes that are specific to melanoma antigens can be identified at tumor sites or in peripheral blood from patients.6 Conversely, an immunosuppressive status often is observed in patients with advanced malignant melanoma,7 and many immunotherapies have been unsuccessful because of such immunosuppression. The number or function of CD4-positive/CD25-positive/forhead box P3 (Foxp3)-positive regulatory T (Treg) cells8-11 or interleukin 10 (IL-10)-producing immunosuppressive dendritic cells12-14 is increased or promoted during the progression of malignant melanoma, even when patients receive some tumor vaccination therapies.5 Investigation of the mechanisms underlying this tumor-induced immunosuppression may provide clues about how to overcome malignant melanoma therapeutically.

Recently, it has been established that programmed cell death-1 (PD-1), an immunoinhibitory receptor that belongs to the CD28 family, plays a critical role in tumor immune escape.15,16 Two ligands for PD-1, PD-1 ligand 1 (PD-L1) and
PD-1 ligand 2 (PD-L2) (also known as B7-DC) are involved in the negative regulation of cellular and humoral immune responses by engaging the PD-1 receptor.15 PD-L1 is expressed on resting T cells, B cells, dendritic cells, macrophages, and parenchymal cells, including vascular endothelial cells and pancreatic islet cells.15,16 Conversely, the expression of PD-L2 is limited to macrophages and dendritic cells.17 Previous studies have demonstrated that PD-1/PD-L interaction inhibits T-cell growth and cytokine secretion18 and that tumor cell-borne PD-L1 induces the apoptosis of tumor-specific T-cell clones in vitro,19 suggesting the potential involvement of PD-Ls in tumor immunity. The expression of PD-L1 in tumors has been reported in melanoma19,20; in cancers of the lung,19 breast,21 ovary,22 kidney,23 pancreas,24 esophageus,25 and bladder;26 and even in adult T-cell leukemia/lymphoma.27 In addition, the involvement of PD-L1 has been demonstrated in the protection of cancer cells from cell lysis by activated T lymphocytes.28 However, the expression of PD-L1 on melanoma cells in relation to tumor cell behavior and prognosis remains to be elucidated. In the current study, we investigated PD-L1 expression in human malignant melanoma to define its clinical significance and relevance to the prognosis for patients with these tumors.

MATERIALS AND METHODS

Patients and Samples

Patients who were enrolled in this study were treated and followed from 2000 to 2007 by the Department of Dermatology, University of Occupational and Environmental Health (Kitakyushu, Japan). Tumors were classified according to the American Joint Committee on Cancer (AJCC) staging system.29 Patients were followed at regular intervals for evaluation of recurrence by physical examination and radiologic studies. Melanoma specimens were collected from 59 primary tumors, 16 lymph nodes (LNs) (9 metastatic LNs and 7 nonmetastatic LNs), and 4 in-transit metastases.

Each specimen was fixed with 20% formalin and embedded in paraffin, and serial sections were stained with hematoxylin and eosin for histologic evaluation. The specimens were digitized by using the NanoZoomer Digital Pathology C9600 (Hamamatsu Photonics, Hamamatsu, Japan), and Breslow tumor thickness (BTT) was analyzed with NDP View software (Hamamatsu Photonics).

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Immunohistochemical staining for PD-L1 was achieved by using a monoclonal antibody (MoAb) capable of detecting PD-L1 on formalin-fixed, paraffin-embedded specimens.22 Sample specimens were cut into 4-mm-thick sections that were deparaffinized in xylene (3 times for 10 minutes each) and dehydrated through graded alcohols.
Antigens were retrieved by boiling in citrate buffer, pH 6.0, using microwaves. To block endogenous peroxidase activity, all sections were treated with 100% methanol containing 0.3% H2O2 for 15 minutes. Nonspecific binding of immunoglobulin G was blocked by using normal rabbit serum (Nichirei, Tokyo, Japan). The sections were incubated with mouse anti-PD-L1 MoAbs (clone 27A2; MBL, Nagoya, Japan) overnight at 4°C. Then, they were incubated with biotinylated rabbit-antimouse secondary antibody (Nichirei) and subsequently incubated in a streptavidin-peroxidase complex solution for 30 minutes. Signals were generated by incubation with 3-amino-9-ethyl carbazole. Finally, the sections were counterstained with hematoxylin.

Analysis of Expression Intensity in Histologic Specimens
Digitized specimens were exported to JPG files by using NDP View software (Hamamatsu Photonics). The following processes were performed in Adobe Photoshop CS (J) (Adobe Systems, Inc. San Jose, Calif). Three different areas from the tumor cell cytoplasm were selected and expressed as Red channel histograms. In the bar graphs that were produced, the horizontal and vertical axes represented tone and quantity, respectively. Histograms revealed 255 different shades from pitch black (0) to pure white (255), and a number represented the level of brightness of each color. We analyzed the mean intensity of the histogram in the cytoplasm and averaged the value of 3 different areas. To obtain density, we calculated the 255-“mean” of each color. We called these values “red density” (RD) values and used them for further investigation. Specimens with an RD value < 90 were defined as the low expression group, and specimens with an RD value ≥ 90 were defined as the high expression group.

Flow Cytometric Analysis
Blood samples were collected from 10 patients who had stage IV melanoma and from 5 normal, healthy volunteers to evaluate PD-1 expression on T cells. Peripheral blood mononuclear cells (PBMCs) were isolated from the heparinized venous blood of patients by using Ficoll-Hypaque (Sigma Chemical Company, St. Louis, Mo) density-gradient centrifugation. Four local metastatic skin lesions were removed surgically, dissociated by teasing, and subjected to flow cytometric analysis. Single cell suspensions were obtained from the excised metastatic skin tumors by teasing and filtering and were subjected to flow cytometric analysis. Cells were double stained with fluorescein isothiocyanate-conjugated anti-PD-1, anti-PD-L1, or anti-PD-L2 MoAb and phycoerythrin (PE)-conjugated anti-CD4 or anti-CD8 MoAb (all from BD Biosciences, San Diego, Calif) at 2 μg/10⁶ cells in Hanks balanced salt solution containing 0.1%
NaN₃ and 1% fetal calf serum. After incubation for 30 minutes at 4°C with MoAbs or isotype-matched controls, cells were washed twice and analyzed on a FACSCanto (Becton Dickinson, Mountain View, Calif). The mean fluorescence intensity (MFI) was calculated on a log scale.

**Statistical Analyses**

Fisher exact tests, chi-square tests, and Student t tests for unpaired data were used to analyze the association between PD-L expression and various clinicopathologic factors. The Pearson coefficient test was used to evaluate the correlation between RD and BTT. Univariate analyses of overall survival and progression-free survival were conducted with the log-rank test, and Kaplan-Meier curves were generated. Overall and progression-free survival was calculated from the date of operation to the date of first recurrence, death, or last follow-up. Multivariate comparisons were made using the Cox proportional hazards model. Except for the Cox multivariate analysis, every analysis was performed using GraphPad Prism 5.0 software (GraphPad Software, Inc., San Diego, Calif). The Cox multivariate analysis was performed using the JMP 5.0.1J software package (SAS Institute, Cary, NC). All P values <.05 were considered statistically significant.

**RESULTS**

**Clinical Patient Profiles**

The clinical characteristics of 59 patients (ratio of men to women, 38:21) are summarized in Table 1 in relation to expression levels of PD-L1 in tumor cells (low and high). The average patient age was 69.47 years (range, 25-87 years; standard deviation, 13 years). The most common site of melanoma was the extremity (78%), followed by the trunk (15.3%), and head and neck (6.8%). Thirty-nine patients were diagnosed with acral lentiginous melanoma, 10 patients were diagnosed with nodular melanoma, 8 patients were diagnosed with superficial spreading melanoma, and 2 patients were diagnosed with lentigo maligna melanoma. According to the AJCC staging system, 29 8 patients (13.6%) had stage 0 melanoma, 6 patients (10.2%) had stage IA melanoma, 9 patients (15.3%) had stage IB melanoma, 8 patients (13.6%) had stage IIA melanoma, 7 patients (11.9%) had stage IIB melanoma, 5 patients (8.5%) had stage IIC melanoma, 3 patients (5.1%) had stage IIIA melanoma, 5 patients (8.5%) had stage IIIB melanoma, 7 patients (11.9%) had stage IIIC melanoma, and 1 patient (1.7%) had stage IV.
melanoma. Ulceration was present in 18 patients (30.6%). LN metastases were observed in 16 patients.

**PD-L1 Expression and Tumor Cell Expression**
The primary tumors from each patient was stained immunohistochemically for PD-L1, and the intensity of its expression level was analyzed as described above (see Materials and Methods). There were 25 patients in the “low-expression” group (RD value, <90) and 34 patients in the “high-expression” group (RD value, ≥90). Representative histopathologic photomicrographs for the high-expression and low-expression groups are provided in Figure 1a and Figure 1b, respectively. The correlations between the PD-L1 expression level and clinical patient profiles are summarized in Table 1. There was no significant correlation between sex and RD or between age and RD. The BTT in the high-expression group was significantly higher than that in the low-expression group (P = .0298). The correlation coefficient between the BTT and the RD value was statistically significant (Fig. 2a), indicating that there was a correlation between PD-L1 expression and the vertical growth of malignant melanoma.

Tumors that were classified as T3-T4 exhibited a significantly higher PD-L1 expression than tumors that were classified as T0-T2 (P = .0072). PD-L1 expression was not associated with ulceration (P = .4031). PD-L1 expression in primary tumors from patients with LN metastasis was significantly higher than that in patients without LN metastasis (P = .0375). Furthermore, PD-L1 expression in metastatic LNs was significantly higher than that in nonmetastatic LNs (Fig. 2b) (mean RD value, 128.7 vs 71.4; P < .0001). Patients with Clark level IV and V tumors expressed significantly higher RD values than patients with Clark level I and II tumors (Fig. 2c).

**Survival and Multivariate Analyses**
The overall survival rate was significantly lower in the PD-L1 high-expression group compared with the low-expression group (Fig. 3a, Table 2) according to Kaplan-Meier survival analyses and log-rank tests. The progression-free survival rate tended to differ between the low-expression and high-expression groups (Fig. 3b, Table 2). Among the other clinicopathologic factors, including the clinical melanoma type (superficial spreading melanoma), primary tumor status,
ulceration, and LN metastasis differed significantly between the 2 expression groups in both overall survival and progression-free survival (Table 2). In patients with stage II melanoma, the high PD-L1 expression group had a significantly lower survival rate than the low-expression group according to log-rank tests (Fig. 3c), and the progression-free survival rate was marginally low without significance in the high-expression group (Fig. 3d). In patients with stage II melanoma, both the low-expression group and the high-expression group exhibited the same BTT levels, but the high-expression group had a significantly lower survival rate. Therefore, we determined that PD-L1 expression is a BTT-independent factor for prognosis.

Multivariate analyses using a Cox proportional hazards model indicated that overall and progression-free survival rates for the high PD-L1 expression group were significantly lower than those for the low-expression group (Table 3). The other factors that contributed to the overall poor survival were primary tumor status, ulceration, and LN metastasis (Table 3), whereas age, sex, and clinical type had no correlation. These data clearly demonstrate that PD-L1 expression in tumor cells is correlated inversely with the prognosis of patients with malignant melanoma and that PD-L1 expression is an independent prognostic factor for both overall and progression-free survival in these patients.

### Elevated PD-1 Expression on T Cells in Stage IV Patients

We evaluated PD-1 expression on CD8-positive and CD4-positive T cells in the peripheral blood from patients with stage IV malignant melanoma. Representative flow cytometric data are shown in Figure 4a, which indicates that there was high expression of PD-1 on CD8-positive T cells and on some populations of CD8-negative cells from a patient with melanoma compared with a normal individual. In all patients that we examined who had stage IV melanoma, both CD8-positive and CD4-positive T-cell populations had significantly higher PD-1 levels.

| Variable                  | No. of Patients | OS RR (95% CI) | P    | OS RR (95% CI) | P    |
|---------------------------|-----------------|----------------|------|----------------|------|
| PD-L1 expression          |                 |                |      |                |      |
| Low                       | 25              | 1              |      | 1              |      |
| High                      | 34              | 3.02 (1.05-8.70)| .0402| 2.52 (0.99-6.44)| .0522|
| Age, y                    |                 |                |      |                |      |
| ≤69                       | 23              | 1              |      | 1              |      |
| ≥70                       | 36              | 0.62 (0.23-1.66)| .3399| 0.64 (0.24-1.70)| .3685|
| Sex                       |                 |                |      |                |      |
| Men                       | 14              | 1              |      | 1              |      |
| Women                     | 9               | 2.44 (0.78-7.65)| .3685| 1.38 (0.51-3.76)| .5292|
| Clinical tumor type       |                 |                |      |                |      |
| ALM                       |                 |                |      |                |      |
| NM                        | 1               | 1              |      | 1              |      |
| SSM                       |                 |                |      |                |      |
| LMM                       |                 |                |      |                |      |
| Primary tumor status      |                 |                |      |                |      |
| pT<sub>T1</sub>-pT2       | 24              | 1              |      | 1              |      |
| pT<sub>T3</sub>-pT4       | 35              | 6.32 (2.01-19.91)|      | 6.66 (1.94-22.89)|      |
| Ulceration                |                 |                |      |                |      |
| Absent                    | 41              | 1              |      | 1              |      |
| Present                   | 18              | 3.98 (1.058-15.01)|      | 3.35 (1.11-10.14)|      |
| LN metastases             |                 |                |      |                |      |
| pN0                       | 44              | 1              |      | 1              |      |
| pN1-pN3                   | 15              | 22.66 (5.776-88.89)|      | 29.32 (7.18-119.8)|      |

OS indicates overall survival; PFS, progression-free survival; RR, risk ratio; CI, confidence interval; PD-1, programmed cell death-1 ligand 1; ALM, acral lentiginous melanoma; NM, nodular melanoma; SSM, superficial spreading melanoma; LMM, lentigo maligna melanoma; pT, pathologic tumor classification; pTis, pathologic tumor in situ; pN, pathologic lymph node status.
compared with the levels in normal, healthy controls (Fig. 4b,c). We also examined PD-1 expression levels in tumor-infiltrating, CD8-positive cells in metastatic skin lesions from 2 patients. The tumor-infiltrating, CD8-positive T cells, as represented by the data from 1 patient obtained at the initial occurrence of melanoma and 3 months later (Fig. 5a), revealed increased expression of PD-1 as the disease progressed. The changes in the degree of PD-1 expression on the CD8-positive T cells from these 2 patients are illustrated in Figure 5b.

**DISCUSSION**

We investigated the expression of PD-L1 in resected specimens from patients with malignant melanoma and observed that there is a correlation between the degree of PD-L1 expression and the vertical growth of primary malignant melanoma. Moreover, our multivariate analysis demonstrated that PD-L1 expression is an independent, poor prognostic factor for malignant melanoma. A representative finding is that the survival rate of the PD-L1 high-expression group was significantly lower than that of the low-expression group with stage II melanoma. Although there has been a report regarding PD-L1 expression on melanoma cells, the clinical significance of PD-L1 in melanoma has not been fully elucidated. Our current study clearly demonstrated the relevance of PD-L1 expression to the growth and prognosis of melanoma cells. The direct involvement of PD-L1 has been demonstrated through the mechanism by which cancer cells escape from the lysis by activated T cells. The expression of PD-Ls on the cell surface of tumor cells, per se, or on antigen-presenting cells in the tumor environment may induce the apoptosis of tumor-reactive T cells through the engagement of PD-1 and, consequently, may promote tumor growth.

Alternative mechanisms underlying the immunosuppression by melanoma cells have been postulated. Patients with melanoma have high serum levels of IL-10, and the number of IL-10–producing monocytes are increased in these patients. It is possible that the elevated

| Table 3. Cox Multivariate Analysis of Independent Risk Factors for Tumor-Specific Death in Patients With Malignant Melanoma |
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| **Variable** | **No. of Patients** | **OS** | **P** | **PFS** |
| **Variable** | **No. of Patients** | **Multivariate RR**<br>(95% CI) | **P** | **Multivariate RR**<br>(95% CI) | **P** |
| PD-L1 expression Low | 25 | 1.00 | .0125 | 1.00 |
| High | 34 | 2.04 (1.15-4.26) | .0364 | 1.67 (1.04-2.95) |
| Age, y ≤70 | 24 | 1.00 | .904 | 1.00 |
| ≥70 | 35 | 0.81 (0.49-1.36) | 0.403 | 0.80 (0.51-1.30) |
| Sex Men | 38 | 1.00 | 1.00 |
| Women | 21 | 1.11 (0.65-1.83) | 0.96 (0.57-1.53) |
| Clinical tumor type ALM | 39 | 1.00 | 1.00 |
| SSM | 8 | 1.58 (0.73-2.97) | .2162 | 1.79 (0.92-3.27) |
| NM | 10 | 1.34 (0.63-2.50) | .4133 | 1.22 (0.57-2.23) |
| LMM | 2 | 1.01 (0.00-2.34) | .9888 | 0.96 (0.23-2.20) |
| Primary tumor status pTis-pT2 | 24 | 1.00 | 1.00 |
| pT3-pT4 | 35 | 4.40 (1.96-18.78) | 2.67 (1.52-5.57) |
| Ulceration Absent | 41 | 1.00 | 1.00 |
| Present | 18 | 1.73 (1.04-3.00) | 1.84 (1.16-3.05) |
| LN metastasis pN0 | 44 | 1.00 | 1.00 |
| pN1-pN3 | 15 | 1.69 (1.02-2.80) | 1.66 (1.04-2.63) |

OS indicates overall survival; PFS, progression-free survival; RR, risk ratio; CI, confidence interval; PD-1, programmed cell death-1 ligand 1; ALM, acral lentigious melanoma; SSM, superficial spreading melanoma; NM, nodular melanoma; LMM, lentigo maligna melanoma; pT, pathologic tumor classification; pTis, pathologic tumor in situ; LN, lymph node; pN, pathologic lymph node status.
production of IL-10 by those cells leads to the immuno-suppression of tumor immunity by inhibiting cytotoxic T cells or tumor antigen-presenting cells. In addition, IL-10 and transforming growth factor-β can be produced by melanoma cells. In another scenario, the melanoma cell also can induce CD25-positive/Foxp3-positive Treg cells. The presence of a high percentage of Treg cells in metastatic LNs also has been reported. Tumor cells spreading into the LN may induce Treg cells, which allow tumor cells to grow locally, and Treg cells may be activated further by unique or shared tumor antigens. A recent finding demonstrated that PD-L1 signaling regulates the conversion of naive, CD4-positive/CD25-negative/Foxp3-negative T cells into FoxP3-positive Treg cells. These findings suggest that PD-L1 on melanoma cells causes immunosuppression by PD-L1–induced Treg cells as well as PD-1/PD-L1 interaction.

PD-1 is expressed on “exhausted” T cells and suppresses immune activation. PD-1 is expressed on post-vaccination, melanoma antigen-specific, cytotoxic T lymphocytes (CTLs). PD-1 blockade during peptide stimulation augmented the absolute numbers of vaccine peptide tetramer-positive CTLs. Our study also demonstrated that PD-1–bearing, CD8-positive cells were increased in PBMCs and in the tumor microenvironment. The number of circulating PD-1–positive/CD8-positive

Figure 4. Programmed cell death-1 (PD-1) expression is illustrated in CD8-positive (CD8+) and CD4+ cell populations in peripheral blood. Peripheral blood mononuclear cells were isolated from the peripheral blood of patients with stage IV melanoma and subjected to flow cytometric analysis. Representative flow cytometric analyses of PD-1 expression on CD8+ cells are illustrated in (a) a patient with stage IV melanoma and (b) a normal, healthy control. PD-1 expression is illustrated (a) on CD8+ cells, (b) on CD4+ cells, and in normal, healthy controls. Error bars represent the mean ± standard deviation. MFI indicates mean fluorescence intensity.

Figure 5. Elevated programmed cell death-1 (PD-1) expression in tumor-infiltrating CD8-positive T cells during tumor progression is shown. (a) PD-1 expression on CD8-positive cells from tumor-infiltrating lymphocytes is illustrated (left) at the initial occurrence of a metastatic skin tumor and (right) 3 months later. The tinted area indicates PD-1 expression; solid line, isotype control. Max indicates maximum. (b) In 2 patients, the alteration of PD-1 expression on CD8-positive cells was monitored. The mean fluorescence intensity (MFI) was calculated as (MFI of PD-1 expression) – (MFI of isotype control).
T cells increased further as the disease progressed. Therefore, it is likely that the exhausted tumor-killing T cells are elevated in number in parallel with the elevation of its ligand on the tumor cell. This dual alternation strongly suggests the clinical importance of PD-L1 expression on melanoma cells. Our findings provide evidence of the clinical relevance of PD-L1 expression. When PD-L1 is highly expressed on tumor cells in biopsy or excised specimens from patients with melanoma, more careful follow-up and management may be required because of the predicted poor prognosis. Although treatments for melanoma are performed on the basis of the stage of this neoplasm, the PD-L1 expression level may be an additional informative item for the consideration of treatments. Furthermore, it is possible that patients who have high PD-L1 expression are refractory to immunotherapies because of their “exhausted” tumoricidal T cells. For example, immunotherapy using tumor-antigenic peptides induces CTLs against tumor cells; however, when CTLs express PD-1, they may be less functional. Likewise, therapy with tumor antigen-specific MoAbs by antibody-dependent cellular cytotoxicity may be ineffective in patients who have high PD-L1 expression.

Malignant melanoma is an immunogenic but immunosuppressive tumor. Our current finding that PD-L1 expression is correlated with tumor proliferation and patient survival indicates the immunosuppressive aspect of melanoma. Many groups of investigators have reported that blockade of the PD-1/PD-L1 interaction promotes tumor immunity. Conversely, there remains a problem regarding PD-1/PD-L1 blockade because of the possible expansion of poorly immunogenic cells. Further studies may be required to clarify the therapeutic effect of PD-1/PD-L1 blockade in malignant melanoma models and clinical trials.

CONFLICT OF INTEREST DISCLOSURES

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