The mechanism of de novo expression of programmed cell death-ligand 1 in squamous cell carcinoma of the lung

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Abstract. Immune checkpoint mechanisms such as the programmed cell death-ligand 1-programmed cell death 1 (PD-L1-PD-1) axis are utilized by tumor cells to evade the cytotoxicity of effector immune cells. However, environmental factors responsible for the expression of PD-L1 on tumor cells remain to be fully elucidated. We hypothesized that an immunological interaction with tumor-infiltrating CD8+ lymphocytes (CD8+ TILs) would contribute to PD-L1 expression in tumor cells. To verify this hypothesis, we examined the effect of interferon-γ (IFN-γ), a cytokine secreted by CD8+ TILs, on PD-L1 expression in pulmonary squamous cell carcinomas in vitro. We also evaluated the expression of PD-L1 and major histocompatibility complex (MHC) class I molecules on tumor cells and CD8+ TILs in squamous cell carcinomas of the lung (n=77) by immunohistochemistry. IFN-γ upregulated PD-L1 expression on pulmonary squamous carcinoma cells, and the reaction was reversible. In cases where which MHC class I molecule-positive tumor cells were dominant (n=72, 93.5%), cases in which PD-L1-positive tumor cells were dominant (PD-L1+ tumor cell-dominant cases; n=45) were more frequently observed than PD-L1-negative tumor cell-dominant cases (n=27) (P=0.006). The number of CD8+ TILs was significantly higher in PD-L1+ tumor cell-dominant cases compared with PD-L1- tumor cell-dominant cases (P=0.005). These data suggest that the de novo expression of PD-L1 on tumor cells is upregulated by IFN-γ secreted from CD8+ TILs upon recognition of the tumor cells with an MHC class I molecule.

Introduction

Non-small cell lung cancer (NSCLC) is the leading cause of cancer-related mortality worldwide (1). New treatments for advanced and recurrent NSCLC have been introduced, including so-called third generation chemotherapeutic agents such as docetaxel, paclitaxel, vinorelbine, and CPT-11, which have contributed to improving patient prognosis (2). Other therapeutic developments including molecular-targeted therapies for driver oncogenes, such as a tyrosine kinase inhibitor targeting mutations of the epidermal growth factor receptor gene and an inhibitor targeting translocations of the anaplastic lymphoma kinase gene, have improved the survival of patients with advanced and recurrent NSCLC over the past decade (3-5). Recently, a new therapeutic strategy, cancer immunotherapy, has emerged to confer clinical benefits for patients.

In some types of cancer immunotherapy, immune checkpoint inhibitors targeting the programmed cell death 1-programmed cell death-ligand 1 (PD-1-PD-L1) axis, exerted a significant durable clinical response for advanced NSCLC (6-9). Several clinical trials revealed that an objective response (including complete and partial responses) was observed in 10.2-18.4% of NSCLC patients (6,9). The immune checkpoint molecule, PD-L1, located on tumor cells, binds to PD-1 on cytotoxic T lymphocytes (CTLs) which attack tumor cells, and induces apoptosis and exhaust CTLs (10). Inhibiting the interaction between PD-1 and PD-L1 prevents CTLs from unresponsiveness, and maintains their cytotoxic activity against tumor cells.

The PD-1-PD-L1 axis is one of the immune check point systems of tumor cells, but the mechanism of PD-L1 expression on tumor cells remains to be fully elucidated. We previously examined PD-L1 expression in pulmonary adenocarcinomas using immunohistochemistry, and reported the heterogeneous distribution of tumor cells with various levels of PD-L1 expression in a tumor tissue section (11). These data suggested
that PD-L1 expression on NSCLC cells may be influenced by environmental factors. PD-L1 gene expression in human peripheral blood monocytes is upregulated by stimulation with interferon-γ (IFN-γ) (12). IFN-γ also induces PD-L1 expression on microvascular endothelial cells (13), while PD-L1 expression on tumor cells is upregulated by IFN-γ secreted from CD8+ T lymphocytes in the tumor-immune cell interaction (14,15). This indicates that PD-L1 expression on NSCLC cells might also be upregulated by IFN-γ released from CTLs following NSCLC cell recognition.

For recognition by CTLs, NSCLC cells must express the major histocompatibility complex (MHC) class I molecule, which interact with T cell receptors (16). However, tumor cells often delete MHC class I molecule expression to evade CTL immune surveillance in a process known as immune editing (17). Indeed, 24.3-80.5% of tumor cells in NSCLC tumor tissue were reported to delete or downregulate the expression of MHC class I molecule (18-22). Such NSCLC cells are less likely to be recognized by CTLs, or to be exposed to IFN-γ released by CTLs; therefore, they are unlikely to express PD-L1. In this context, we hypothesized that the de novo expression of PD-L1 on NSCLC cells might be associated with CTL infiltration into tumor tissue and the expression of MHC class I molecules on NSCLC cells.

In this study, we focused on the interaction between NSCLC cells and CD8+ TILs in a tumor microenvironment, and aimed to clarify the mechanism of de novo expression of PD-L1 on squamous cell carcinomas of the lung. The data presented here suggest that de novo PD-L1 expression on tumor cells is upregulated by IFN-γ secreted from CD8+ TILs following the recognition of tumor cells with MHC class I molecules.

Materials and methods

Cells. Human pulmonary squamous cell carcinoma cell lines LK-2 and EBC-1 (obtained from the Japanese Collection of Research Bioresource, Tokyo, Japan) were maintained in a humidified atmosphere of 5% CO2 at 37°C. RPMI-1640 medium was used for LK-2 culture, and EBC-1 cells were cultured in Dulbecco’s modified Eagle’s medium, each supplemented with 10% fetal bovine serum.

Flow cytometry. To examine the dose-dependent effect of IFN-γ on surface expression of PD-L1 on tumor cells, the cells were cultured in medium containing 0, 31.25, 125 or 500 pg/ml recombinant human IFN-γ (PeproTech, Rocky Hill, NJ, USA) for 48 h. To examine the reversibility of IFN-γ-induced PD-L1 expression, the cells were cultured in the medium containing 1.0 ng/ml IFN-γ for 48 h. The culture medium was then replaced by fresh medium without IFN-γ, and cell culture was continued for an additional 48 h. Those cells were then harvested and stained with a phycoerythrin (PE)-labeled anti-human PD-L1 antibody (clone: MIH1) (BD Biosciences, San Jose, CA, USA) or a mouse IgG1, k isotype control (BD Biosciences). To examine IFN-γ receptor 1 expression on tumor cells, the cells were stained with a PE-labeled anti-human IFN-γ receptor 1 (CD119) antibody (clone: GIR-208) (eBioscience). Flow cytometric analysis was performed using BD FACSCalibur, and the data were analyzed using BD CellQuest Pro software (BD Biosciences).

Reverse transcription polymerase chain reaction (RT-PCR). Total RNA was extracted from tumor cells using RNasey mini kit (Qiagen, Hilden, Germany), and was subjected to the reverse transcription reaction using cloning AMV first-strand cDNA synthesis kit (Life Technologies, Carlsbad, CA, USA). Each cDNA was amplified with specific paired primers as follows: PD-L1 forward, 5'-GAGCTTCAAGGCAAA TCATC-3'; reverse, 5'-GCAACCAAGGTGTTCTTT-3'. GAPDH forward, 5'-TGGAAAGACTCATGACCACA-3'; reverse, 5'-CCCTGTGTCTGGTACCAAA-3'.

Tumor samples. Tumor samples were obtained from surgically resected specimens of patients with squamous cell carcinoma of the lung at Shiga University of Medical Science Hospital, between January 2008 and December 2012. Patient clinicopathological data were obtained from medical records. The study design was approved by the Ethics Committee of Shiga University of Medical Science (no. 25-225), and a written informed consent was obtained from all the patients.

Immunohistochemistry. Serial sections (4-μm-thick) of formalin-fixed paraffin-embedded tissue specimens were stained by standard indirect immunoperoxidase procedures for PD-L1, CD8, and MHC class I molecules, according to the manufacturer’s protocols. Briefly, each tissue section was deparaffinized in xylene, and rehydrated in ethanol and distilled water. Antigen retrieval was performed by microwave treatment in 10 mM sodium citrate buffer (pH 6.0) for PD-L1 or 10 mM Tris/1 mM ethyleneaminetetraacetic acid (pH 9.0) for CD8 and MHC class I molecules for 10 min. Endogenous peroxidase activity was blocked by treatment with 3% H2O2 for 10 min. After blocking with 5% normal goat serum in Tris-buffered saline with Tween-20 for 1 h at room temperature, the sections were incubated overnight with an anti-human PD-L1 monoclonal antibody (clone: EIL3N, diluted at 1:200) (Cell Signaling Technology, Danvers, MA, USA), an anti-human CD8 antibody (clone: CD8/144B, diluted at 1:200) (Dako, Carpinteria, CA, USA), or an anti-HLA class I molecule antibody (clone: E8R5-5, diluted at 1:500) (Hokudo, Sapporo, Japan) at 4°C overnight. The sections were then incubated with SignalStain boost IHC detection reagent (Cell Signaling Technology) for PD-L1 or Envision Dako ChemMate for CD8 and MHC class I molecules. They were visualized using the SignalStain DAB substrate kit (Cell Signaling Technology) for PD-L1 or Envision Dako ChemMate/HRP (DAB) for CD8 and MHC class I molecules for 1 min, followed by counterstaining with hematoxylin. As an isotype-matched control antibody, rabbit IgG monoclonal antibody (Cell Signaling Technology) was used for PD-L1 immunohistochemistry, and mouse IgG monoclonal antibody (Dako) were used for MHC class I and CD8 immunohistochemistry. In PD-L1 immunohistochemistry, paraffin-embedded cell-blocks of PD-L1-positive H-1975 tumor cells and PD-L1-negative A549 tumor cells were utilized for positive and negative controls, respectively, as described in a previous report (11).

Evaluation of PD-L1, CD8, and MHC class I expression. Serial sections of stained tumor tissue were independently examined by two researchers, including a pathologist. To compare the staining intensities of PD-L1, CD8, and MHC class I mole-
molecules on each tumor cell, the cells which commonly existed in the serial section were evaluated. Under ×200 magnification, three representative fields of view were selected, and 30 tumor cells per field were observed for the expression of PD-L1 and MHC class I molecules. If the frequency of PD-L1-positive tumor cells was >50%, we described it as a PD-L1+ tumor cell-dominant case. If the frequency of MHC class I molecule-positive tumor cells was >80%, we described it as an MHC class I+ tumor cell-dominant case. For an evaluation of TILs, the number of CD8-positive lymphocytes was counted in the fields.

Statistical analysis. The statistical analysis between groups was determined by Chi-square test, P-values <0.05 were considered statistically significant. All analyses were performed using SPSS Statistics 22.0 software (IBM, Armonk, NY, USA).

Results

Patient characteristics. In total, 77 patients with squamous cell carcinomas of the lung were included in this study (Table I). The median patient age at the time of surgery was 71 years (range,
The patients consisted of 72 males (93.5%) and five females (6.5%); 74 patients (96.1%) had a smoking habit.

Postoperative pathological stages of squamous cell carcinoma of the lung were IA in 23 cases (29.8%), IB in 13 (16.9%), IIA in 11 (14.3%), IIB in 13 (16.9%), IIA in 14 (18.2%), IIB in one (1.3%), and IV in two (2.6%). Thirty-three patients (42.9%) received adjuvant chemotherapy for approximately one month after surgery.

Effect of IFN-γ on PD-L1 expression in pulmonary squamous carcinoma cells. Several studies have previously reported that IFN-γ induces PD-L1 expression in human cancer cell lines in vitro, such as pulmonary adenocarcinomas (A549 cells) (23), hepatocytes (HEPG2 cells) (24), and ovarian cancer (SK-OV-3, ovary 1847, and OVCAR8 cells) (25). We examined the effect of IFN-γ on PD-L1 expression in the pulmonary squamous cell carcinoma cell lines Lk2, and EBC-1. Flow cytometry demonstrated that PD-L1 expression on those cell lines were upregulated by IFN-γ exposure in a dose-dependent manner (Fig. 1A). Furthermore, the PD-L1

<table>
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<th>Pathological stage, n (%)</th>
<th>IA</th>
<th>IB</th>
<th>IIA</th>
<th>IIB</th>
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<th>IIIB</th>
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<td>13</td>
<td>11</td>
<td>13</td>
<td>14</td>
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<td>2</td>
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<tr>
<td>IB</td>
<td>29.8</td>
<td>16.9</td>
<td>14.3</td>
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<td>18.2</td>
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<td>2.6</td>
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<tr>
<td>Pathological stage, n (%)</td>
<td>Ia</td>
<td>IB</td>
<td>IIA</td>
<td>IIB</td>
<td>IIIA</td>
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<td>IV</td>
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Table I. The patient characteristics (n=77).

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<td>Sex, n (%)</td>
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<tr>
<td>Male</td>
<td>72 (93.5)</td>
</tr>
<tr>
<td>Female</td>
<td>5  (6.5)</td>
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<tr>
<td>Smoking status, n (%)</td>
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<tr>
<td>Current/former</td>
<td>74 (96.1)</td>
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<td>Never</td>
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Figure 2. Immunohistochemical analysis of MHC class I molecules and PD-L1 expression in squamous cell carcinomas of the lung. (A) Representative data of MHC class I+ and PD-L1+ tumor cell-dominant cases (n=45, 58.4%). (B) MHC class I- and PD-L1- tumor cell-dominant cases (n=5, 6.5%). (C) MHC class I+ and PD-L1- tumor cell-dominant cases (n=27, 35.1%). No MHC class I- and PD-L1+ tumor cell-dominant cases were observed in this study (n=0, 0.0%). Scale bar, 100 µm.
expression induced by IFN-γ was downregulated by depletion of IFN-γ from the culture medium (Fig. 1B), demonstrating that PD-L1 expression on the tumor cells was reversible. Next, we examined whether levels of PD-L1 mRNA were affected by IFN-γ. The levels of PD-L1 mRNA in those cell lines were also positively and reversibly regulated by IFN-γ (Fig. 1C). In addition, we evaluated IFN-γ receptor expression on those tumor cells. Flow cytometry revealed that expression intensity of IFN-γ receptor 1 was higher in Lk2 than EBC-1 (Fig. 1D). The mean fluorescence intensity of PD-L1-expressed tumor cells increased 1.7 and 2.0 times by IFN-γ in Lk2 and EBC1, respectively (Fig. 1B). In the RT-PCR, signal intensity of PD-L1 mRNA increased 1.4 and 1.8 times by IFN-γ in Lk2 and EBC1, respectively. We detected the expression of IFN-γ receptor 1 on these cells, however these levels were less likely to affect the level of PD-L1 expression induced by IFN-γ. These data demonstrate that PD-L1 expression in pulmonary squamous carcinoma cells is reversibly regulated by IFN-γ.

Expression of PD-L1 and MHC class I molecules in squamous cell carcinoma of the lung. TILs appear to be the main source of IFN-γ in the tumor microenvironment, and their secretion of IFN-γ occurs after recognition of MHC class I molecule expression by tumor cells (26). On the basis of this finding, we examined the correlation between the expression of PD-L1 and MHC class I molecules by tumor cells (Fig. 2). The median frequency of PD-L1+ tumor cells was 55.1% (range, 0.0-98.1%), and 45 PD-L1+ tumor cell-dominant cases compared with MHC class I tumor cell-dominant cases (45/72, 62.5% vs. 0/5, 0%, P=0.006) (Fig. 3). In contrast, in MHC class I tumor cell-dominant cases (n=5), no PD-L1+ tumor cell-dominant case was observed, and all cases were PD-L1 tumor cell-dominant (Fig. 2). These data suggest that the expression of MHC class I molecule is needed for the de novo expression of PD-L1 on tumor cells.

**Figure 3.** Correlation between the expression of MHC class I molecules and PD-L1 in squamous cell carcinomas of the lung. The frequency of PD-L1+ tumor cell-dominant cases in MHC class I+ and MHC class I- tumor cell-dominant cases. The frequency of PD-L1+ tumor cell-dominant cases was significantly higher in MHC class I+ tumor cell-dominant cases compared with MHC class I tumor cell-dominant cases (45/72, 62.5% vs. 0/5, 0%, P=0.006).

**Correlation between PD-L1 expression and CD8+ TILs in squamous cell carcinomas of the lung.** Even in tumor cells expressing MHC class I molecule, a few CTLs are unlikely to contribute to the expression of PD-L1. In this context, we examined the correlation between PD-L1 expression on tumor cells and CD8+ TILs in MHC class I+-dominant cases (Fig. 4A and B). We observed median numbers of CD8+ TILs of 84.5 for PD-L1+ tumor cell-dominant cases (range, 3.3-569.7), and 73.7 for PD-L1+ tumor cell-dominant cases (range, 40.7-135.0). Significantly higher numbers of CD8+ TILs were shown to migrate to the tumor tissues of PD-L1+ tumor cell-dominant cases compared with PD-L1+ tumor cell-dominant cases (P=0.005) (Fig. 4C). These data suggest that the interaction between MHC class I-expressing tumor cells and abundant CD8+ TILs contributes to the de novo expression of PD-L1 on tumor cells.

**Discussion**

In this study, we present the potential mechanism of de novo PD-L1 expression in squamous cell carcinomas of the lung. PD-L1 expression is reversibly regulated by IFN-γ in vitro, and in a tumor microenvironment, it is associated with the expression of MHC class I molecule on tumor cells and the number of CD8+ TILs. These data suggest that CD8+ TILs secrete IFN-γ into the tumor microenvironment following an interaction with MHC class I-positive tumor cells, leading to the upregulation of PD-L1 expression on tumor cells.

Tumor cells have been reported to acquire mechanisms to evade the immune surveillance system of antitumor immune cell types, leading to tumor progression (27). Among these immune escape systems, the deletion of MHC class I molecules on tumor cells prevents CTLs from recognizing them through the interaction with T cell receptors (17). In the present study, tumor cells with deleted MHC class I molecule ranged in frequency from 8.8 to 100% (median, 88.7%), and the frequency of cases in which MHC class I molecule was expressed on almost all tumor cells was found to be 66.2% (52/77). In five cases (6.5%), tumor cells with deleted MHC class I molecule dominated the tumor tissue. In these cases, CTLs activated following immunotherapy would exert no cytotoxic effect on tumor cells because of few chances of recognition. Therefore, a case in which the frequency of tumor cells with deleted MHC class I molecule is low would be a good candidate for immune checkpoint inhibitors.

IFN-γ is the most potent factor for PD-L1 expression in a tumor microenvironment (28,29). After IFN-γ binding to its receptor on tumor cells, it induces signal transducer and activator of transcription 1 activation via the mitogen activated protein kinase/extracellular signal-regulated kinase pathway (30). In the present study, flow cytometry revealed that PD-L1 expression on tumor cells was upregulated following IFN-γ exposure for 48 h in a dose-dependent manner. Interestingly, PD-L1
expression was downregulated 48 h after the removal of IFN-γ from culture medium, demonstrating that the PD-L1 response to IFN-γ was reversibly regulated. These data suggest that the PD-L1 status of tumor cells is affected by the level of IFN-γ in the tumor microenvironment, and is constantly changing. Previously, we and others reported heterogeneity in levels of PD-L1 expression between NSCLC cells within a section of tumor tissue (11,31,32). We propose that this reflects the distinct levels of IFN-γ in the tumor microenvironment which affect PD-L1 expression.

When considering the cell type of the main source of IFN-γ secretion into the tumor microenvironment, IFN-γ is usually released from CTLs following tumor cell recognition (26); thus, IFN-γ secreted by CTLs might affect PD-L1 expression on NSCLC cells. Given that MHC class I molecule on tumor cells are required for their recognition by CTLs, we examined the association between expression of PD-L1 and that of MHC class I molecule on NSCLC cells. In all cases where NSCLC cells with deleted MHC class I molecules were dominant (n=5), PD-L1 expression was significantly downregulated. This is to be expected because such NSCLC cells are not recognized by CTLs, so do not receive stimulation by IFN-γ released from CTLs, ultimately leading to PD-L1 downregulation.

We also examined the association between expression of PD-L1 and that of MHC class I molecule in cases where NSCLC cells expressing MHC class I molecule were dominant. We showed that higher PD-L1 expression on NSCLC cells was significantly associated with an increased number of CD8+ TILs. These data suggest that PD-L1 expression cannot be upregulated without sufficient stimulation from IFN-γ released by CTLs, even in NSCLC cells expressing MHC class I molecule. However, several factors or conditions, such as enhanced PD-L1 expression by some altered signals derived from epidermal growth factor receptor (EGFR) mutation (33), Kras mutation (34), and echinoderm microtubule-associated protein-like 4 (EML4) - anaplastic lymphoma kinase (ALK) rearrangement (35), may be additively regulating the PD-L1 status of tumor cells. The data presented in Fig. 4C also suggest that other mechanisms than IFN-γ-induced de novo PD-L1 expression may be involved in PD-L1 status of tumor cells. Although the level of IFN-γ in the tumor microenviron-
ment should be investigated further, de novo PD-L1 expression in NSCLC cells expressing MHC class I molecule seems to be upregulated by IFN-γ released from CD8+ TILs following NSCLC cell recognition.

In the treatment of NSCLCs by immune checkpoint inhibitors such as anti-PD-L1/PD-1 antibodies, predictive biomarkers of a clinical response have yet to be established. Several studies have suggested that immune checkpoint inhibitors could be possible biomarkers. In PD-L1 inhibition, a clinical response was associated with PD-L1 expression on tumor-infiltrating immune cells such as macrophages, dendritic cells, and T cells (36,37). Moreover, in anti-PD-1 therapy, an association between the therapeutic response and PD-L1 expression on tumor cells was reported (38). On the basis of the present data, we propose that the status of MHC class I molecule expression on NSCLC cells could be used as a predictive biomarker for immune checkpoint inhibitors as well as other types of cancer immunotherapies.

Even if antitumor immune responses are improved by immunotherapy, NSCLC cells with deleted MHC class I molecule would not be attacked by an improved CTL response. Therefore, the status of MHC class I molecule expression on NSCLC cells should be determined prior to immunotherapy. Because we suggest that both the expression of MHC class I molecule on NSCLC cells and a sufficient number of CD8+ TILs are required for the de novo expression of PD-L1 on tumor cells, many CD8+ lymphocytes will infiltrate the tumor tissue when it is dominated by PD-L1-positive tumor cells. In such cases, a PD-L1/PD-1 blockade would be an efficient means of improving the antitumor immune response.

In conclusion, PD-L1 expression in pulmonary squamous cell carcinomas appears to be reversibly regulated by IFN-γ in vitro. In cases of squamous cell carcinomas of the lung in which MHC class I molecule-positive tumor cells are dominant, the high frequency of PD-L1-positive tumor cells is associated with high numbers of CD8+ TILs. This suggests that the de novo expression of PD-L1 on MHC class I molecule-positive tumor cells is upregulated by IFN-γ secreted by CD8+ TILs after tumor cell recognition via MHC class I molecules.

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