B7-H1 Expression on Non-Small Cell Lung Cancer Cells and Its Relationship with Tumor-Infiltrating Lymphocytes and Their PD-1 Expression

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ABSTRACT

Purpose: B7-H1/PD-L1 (B7-H1) and B7-DC/PD-L2 (B7-DC) are ligands for the receptor PD-1, which is known to negatively regulate T-cell activation. In the present study, we investigated the expression of B7-H1 and B7-DC in tumor specimens of non-small cell lung cancer and their relationships with clinicopathological variables and postoperative survival. Furthermore, we examined the correlation between B7-H1 expression on tumor cells and the number of tumor-infiltrating lymphocytes (TILs) or PD-1 expression on TILs.

Experimental Design: The expression of B7-H1 and B7-DC was focally observed in all non-small cell lung cancer tumor specimens. No relationship was found between the expression of B7-H1 or B7-DC and clinicopathological variables or postoperative survival. However, in the same sections evaluated, significantly fewer TILs were identified in B7-H1-positive tumor regions than in B7-H1-negative tumor regions in a subset of five patients (P = 0.01). Moreover, the percentage of TILs expressing PD-1 was significantly lower in B7-H1-positive tumor regions than in B7-H1-negative tumor regions (P = 0.02).

Conclusions: The expression of B7-H1 on tumor cells in local areas reciprocally correlated with the number of TILs, and this may contribute to negative regulation in antitumor immune responses in non-small cell lung cancer.

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INTRODUCTION

Lung cancer is one of the most common fatal malignancies, and the incidence of this type of cancer is increasing worldwide. Platinum-based chemotherapy and radiation have low efficacy against lung cancer due to frequent recurrence and metastasis of the neoplasm; the overall 5-year survival rate after diagnosis remains at 10–15% (1). To improve the poor prognosis of lung cancer patients, studies of new therapeutic strategies including immunotherapy are in progress.

Effective protective immunity against cancer depends on the concordant activity of CTLs (2). T-cell activation is the result of a balance between positive and negative signals. CD28 and ICOS are positive costimulatory receptors interacting with the ligands of the B7 family on professional antigen-presenting cells and are essential for activation and proliferation of antigen-specific T cells (3). In contrast, negative signals through cell surface molecules such as CTLA-4, CD95, CD5, CD31, LAIR, Ly49A, and NKG2A inhibit T-cell activation or induce apoptosis (4).

B7-H1/PD-L1 (B7-H1) and B7-DC/PD-L2 (B7-DC) are members of the B7 superfamily (5, 6). B7-H1 and B7-DC share 40% amino acid homology and are more homologous to each other than to other ligands of the B7 family (7). These B7 family members have been shown to down-regulate T-cell activation through receptor PD-1 (6, 8, 9). Cross-linking of PD-1 by B7-H1 or B7-DC results in decreased interferon γ, interleukin (IL)-10, IL-4, and IL-2 secretion (6, 8). Thus, on T-cell receptor activation, B7-H1 or B7-DC leads to diminished immune responses, and the two molecules may have overlapping functions.

PD-1, which has been identified as a receptor for B7-H1 and B7-DC, belongs to the CD28/CTLA-4 subfamily of the immunoglobulin superfamily and contains tyrosines in ITIM-like motifs that may recruit phosphatases, similar to other negative regulators (7, 10). PD-1−/− mice display a variety of autoimmune diseases, demonstrating the role of PD-1 as a negative regulator of the immune response (10, 11).

B7-H1 and B7-DC are more broadly expressed than the other B7 superfamily members. Initial studies documented the expression of B7-H1 and B7-DC in mRNA in nonlymphoid organs as well as lymphoid organs (5, 6, 8). Recent studies at the protein level have revealed that B7-H1 is expressed on the endothelium in the thymus, heart, and placenta in both humans and mice (12–14) in addition to lymphoid cells, such as activated T cells, B cells, macrophages, and dendritic cells. B7-DC protein is also expressed in the thymus, placenta and heart in both humans and in the thymus in mice (12, 14). However, B7-DC expression is more restricted on professional antigen-presenting cells, such as activated monocytes and dendritic cells (5, 6, 8). On the other hand, PD-1 protein is expressed on double-positive...
and -negative thymocytes, activated T and B cells, and myeloid cells (9, 10, 14, 15).

B7-H1 is also abundant on tumor cell lines and tumor tissues, including lung carcinomas, ovarian carcinomas, breast carcinomas, glioblastoma, and squamous cell carcinoma of the head and neck (12, 13, 16, 17). Although B7-DC expression has been noted in several murine tumor cell lines (6), little is known about human B7-DC expression in tumor tissue. Cancer cells expressing B7-H1 have been shown to increase apoptosis of antigen-specific human T-cell clones (13) and to inhibit CD4+ and CD8+ T-cell activation in vitro (16). In addition, mice succumb to tumors transfected with B7-H1 even after adoptive T-cell immunotherapy, whereas blockade of PD-1/B7-H1 inhibits tumorigenesis in vivo (17, 18). However, the functional roles of tumor-related B7-H1 and association of PD-1 with B7-H1 have not been analyzed previously in human tumor tissue.

In the present study, using immunohistochemistry, we investigated the extent of B7-H1 and B7-DC expression in tumor specimens of non-small cell lung cancer, and we analyzed the relationship between their expression and clinicopathological variables or postoperative survival. Furthermore, we also examined the association between B7-H1 expression on tumor cells and PD-1 expression on tumor-infiltrating lymphocytes (TILs).

PATIENTS AND METHODS

Tumor Specimens and Survival Data. Primary tumor specimens were obtained by surgery from 52 non-small cell lung cancer patients (35 men and 17 women; mean age at diagnosis, 66.4 years) at Hokkaido University Medical Hospital, Japan, between 1997 and 2003. Surgically resected specimens were fixed in formalin and embedded in paraffin for routine histopathological diagnosis and embedded in OCT compound (Miles Laboratories, Elkhart, IN) and snap frozen in liquid nitrogen for immunohistochemical analysis. The surgically resected specimens included 31 adenocarcinomas and 21 squamous cell carcinomas, based on World Health Organization criteria of histopathological classification (19). The tumors were classified as stage I (n = 35), stage II (n = 11), stage III (n = 5), and stage IV (n = 1) tumors based on the American Joint Committee on Cancer guidelines for postsurgical tumor-node-metastasis (20). No patient underwent radiation or chemotherapy before surgery. Survival of the 52 non-small cell lung cancer patients was analyzed for patients who met the following criteria: (a) survived for >3 months after surgery; and (b) did not die of any cause other than lung cancer after surgery. Two patients who did not meet the above-mentioned criteria were excluded from the survival analysis; one died within 3 months after surgery, and the other patient died due to a cause other than lung cancer.

Immunohistochemistry. First, 4–5-μm sections of the specimens were air-dried for 10 min and then fixed in acetone for 10 min. Endogenous peroxidase activity was blocked by 0.3% hydrogen peroxidase in PBS for 30 min. Sections were then washed three times in PBS. After blocking nonspecific binding with serum (Vectastain ABC kits; Vector Laboratories, Burlingame, CA) for 20 min, sections were incubated with the primary antibodies in a humid chamber at 4°C overnight. Anti-B7-H1 (MIH1), anti-B7-DC (MIH14), and anti-PD-1 (MIH4) antibodies (diluted 1:200; Ref. 21) and anti-CD45 antibody (UCHL-1; DAKO, Carpinteria, CA; diluted 1:100) were used as
the primary antibodies. After three washes with PBS, sections were incubated with biotinylated secondary antibodies for 30 min, washed three times in PBS, and incubated with streptavidin-conjugated peroxidase for 30 min. After three additional washes in PBS, 3,3′-diaminobenzidine tetrahydrochloride was applied, and sections were then counterstained with hematoxylin. The entire procedure, with the exception of incubation with primary antibodies, proceeded at room temperature. Nonimmunized mouse IgG for B7-H1, B7-DC, and PD-1 was substituted for the primary antibody in the negative controls.

Cell Counting. B7-H1 and B7-DC expression was defined as the percentage of tumor cells displaying immunoreactivity in the cytoplasm or on the membrane and calculated by counting the number of B7-H1- and B7-DC-stained tumor cells among 1000 tumor cells in each section. One or two representative tissue sections were taken from each tumor, and whole areas were surveyed microscopically at ×100 magnification. Cell counts were performed at ×400 in at least five fields in randomly selected tumor areas.

To examine whether B7-H1 expression was associated with infiltration of TILs and PD-1 expression of TILs, we quantified the infiltration of CD45+ cells and PD-1 expression of these cells in PD-L1-positive and -negative non-small cell lung cancer tumor regions, as described previously (22). First, B7-H1-positive and B7-H1-negative areas were located on a B7-H1-stained tumor section. Consecutive slides from the same tumor, stained for either CD45 or PD-1, were superimposed on the B7-H1-stained slide. Using histological landmarks, the corresponding B7-H1-positive and B7-H1-negative areas were located on these slides. The B7-H1-stained section was removed, and a second investigator, who had no prior knowledge of the local status of B7-H1, counted the number of CD45+ cells per 1000 total nuclei or the number of PD-1+ CD45+ cells per 500 total CD45+ cells. All of the counting was done in a blinded fashion; the observers (J. K. and K. Y.) were not informed of the outcome of the patients or the results of other observers. For analysis of relationships with clinicopathological variables or survival time, patients were divided into two groups using the median number of B7-H1-positive cells or B7-DC-positive cells as the distinguishing factor.

Statistical Analysis. The association between the number of immunoreactive cells and clinicopathological variables was analyzed statistically using Student’s paired t test, Wilcoxon’s rank-sum test, or the χ² test, as appropriate, using Statview software version 4.5 (SAS Institute). The correlation between the percentage of B7-H1 and B7-DC was analyzed statistically using Spearman’s rank correlation. The survival curves were estimated by the Kaplan-Meier method. Values of \( P < 0.05 \) were considered to indicate statistical significance, and all tests were two-tailed.

RESULTS

B7-H1 and B7-DC Expression on Tumor Cells. Among all 52 surgically resected specimens of non-small cell lung cancer without preoperative therapy, the expression of B7-H1 and B7-DC was demonstrated in the cell membrane, cytoplasm, or both, in a focal or scattered pattern (Fig. 1). The percentage of B7-H1-positive cells in non-small cell lung cancer was 27.2 ± 4.7% (mean ± SD; median, 11.2%), whereas the percentage of B7-DC-positive cells was 11.4 ± 2.6% (mean ± SD; median, 7.9%). The percentage of B7-H1-positive cells in adenocarcinoma was larger (mean ± SD, 27.6 ± 33.2%; median, 12.1%) than that in squamous cell carcinoma (mean ± SD, 26.5 ± 35.2%; median, 7.9%). On the other hand, the percentage of B7-DC in squamous cell carcinoma was higher (mean ± SD, 13.1 ± 19.4%; median, 10.5%) than that in adenocarcinoma (mean ± SD, 10.6 ± 18.5%; median, 7.5%). There was a statistically significant correlation between the percentage of B7-H1-positive cells and that of B7-DC-positive cells in both adenocarcinoma (\( R^2 = 0.23; y = 0.266x + 3.267; P < 0.01; \) Fig. 2A) and squamous cell carcinoma (\( R^2 = 0.53; y = 0.397x + 2.01; P < 0.01; \) Fig. 2B). However, concomitant expression of B7-H1 and B7-DC was rarely observed on the same tumor cells (Fig. 3).

Next, we divided the 52 non-small cell lung cancer patients who had resected tumors into two groups to analyze correlation between B7-H1 or B7-DC expression and clinicopathological variables, using the median number of the percentages of B7-H1-positive cells and B7-DC-positive cells as the distinguishing factor. Tables 1 and 2 show the relationships between expression of either protein and clinicopathological variables. No correlation was observed between B7-H1 or B7-DC expression and either age, sex, smoking habit, histology, differentiation, pT
classification, pN classification, or pStage classification. Among the 50 non-small cell lung cancer patients with potentially resected tumors, no relationship was found between B7-H1 or B7-DC expression and patient survival [5-year survival rates: 59% in those with B7-H1-positive non-small cell lung cancer and 48% in those with B7-H1-negative non-small cell lung cancer (P = 0.89); 53% in those with B7-DC-positive non-small cell lung cancer and 50% in those with B7-DC-negative non-small cell lung cancer (P = 0.43)]. Although the survival of 10 patients with higher expression of B7-H1 or B7-DC was compared with 10 corresponding patients with no expression of either relevant molecule, no significant differences in either analysis were observed.

**Decreased Infiltration of TILs with Decreased PD-1 Expression in B7-H1-Expressing Tumor Regions.** All non-small cell lung cancers contained infiltrates of cells that were immunohistochemically positive for CD45 (leukocyte common antigen) and almost exclusively of lymphoid morphology, indicating that they were TILs (Fig. 4). To evaluate whether B7-H1 or B7-DC expression on non-small cell lung cancers limited immune effector cell infiltration within tumors, we counted and compared the TILs in B7-H1- or B7-DC-positive and -negative tumor regions on the same sections. Of all non-small cell lung cancers examined, only five contained separate B7-H1-positive and -negative tumor regions in the same sections. Because the other samples had B7-H1-positive and -negative tumor cells mixed together in the same tumor nests throughout the sections and B7-H1-positive and -negative tumor regions were not separated, no comparison was possible (Fig. 1C). On the other hand, all non-small cell lung cancer samples had B7-DC-positive and -negative tumor cells mixed together in the same tumor nests throughout the sections, and the association between B7-DC expression on non-small cell lung cancers and immune effector cell infiltration within tumors also could not be analyzed (Fig. 1D). In the five sections that were suitable for analysis, the amount of TIL infiltration was significantly reduced in B7-H1-expressing tumor regions (Fig. 4). B7-H1 expression was associated with a statistically significant (on average, 2.5× reduction) in TILs in B7-H1-positive tumor regions compared with B7-H1-negative tumor regions within the same sections (22.6 ± 13% and 51.5 ± 14.1%, respectively; P = 0.01; Table 3). On the other hand, there were no histological or clinicopathological differences compared with the rest of tissues. The percentage of B7-DC-positive cells in these five

### Table 1 Correlation between B7-H1 expression on tumor cells and the clinicopathological characteristics of the 52 non-small cell lung cancers

<table>
<thead>
<tr>
<th>B7-H1 expression on tumor cells</th>
<th>Low (&lt;11%)</th>
<th>High (≥11%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients</td>
<td>26</td>
<td>26</td>
<td>0.28*</td>
</tr>
<tr>
<td>Age (mean ± SD) (yrs)</td>
<td>64.9 ± 1.7</td>
<td>68 ± 2.1</td>
<td></td>
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<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>20</td>
<td>15</td>
<td>0.14†</td>
</tr>
<tr>
<td>Female</td>
<td>6</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>Smoking habits</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Smoker</td>
<td>18</td>
<td>19</td>
<td>NS†</td>
</tr>
<tr>
<td>Nonsmoker</td>
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<td>7</td>
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</tr>
<tr>
<td>Histology</td>
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<td></td>
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</tr>
<tr>
<td>SCC</td>
<td>14</td>
<td>17</td>
<td>0.4†</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
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</tr>
<tr>
<td>Differentiation</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Well</td>
<td>10</td>
<td>5</td>
<td>0.13†</td>
</tr>
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<td>16</td>
<td>21</td>
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<tr>
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<td>8</td>
<td>6</td>
<td>0.54†</td>
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<td>15</td>
<td>0.78†</td>
</tr>
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<td>N1-3</td>
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<td>pStage classifications</td>
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</tr>
<tr>
<td>I</td>
<td>13</td>
<td>12</td>
<td>0.78†</td>
</tr>
<tr>
<td>II-IV</td>
<td>13</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>CD45+ cells†</td>
<td>47.8 ± 3.2</td>
<td>42.8 ± 3.1</td>
<td>0.27*</td>
</tr>
</tbody>
</table>

**Abbreviations:** NS, nonsignificant; SCC, squamous cell carcinoma.  
* Student’s paired t test.  
† χ² test.  
‡ Percentage of CD45-positive cells per 1000 total nuclei.
sections was 15 ± 3.5% (mean, 12.5%), and there was no difference compared with that in the rest of tissues (P = 0.65).

Moreover, we analyzed the expression of PD-1, one of the receptors of B7-H1, on TILs in B7-H1-positive tumor regions and in B7-H1-negative regions. A statistically significant (on average, 3-fold) decrease in PD-1-positive CD45-positive TILs was observed in B7-H1-positive tumor regions compared with B7-H1-negative tumor regions (7.1 ± 4.5% and 20.2 ± 9.0%, respectively; P = 0.02; Table 3).

### DISCUSSION

We have demonstrated the expression of B7-H1 and B7-DC in surgically resected specimens of non-small cell lung cancer, and we have demonstrated that the expression of these molecules is found in both the plasma membrane and cytoplasm of cancer cells. The expression patterns of these molecules were consistent with previous reports, which examined their expression in human tumor tissue (12, 13, 16, 17). The present study...
also examined the relationship between the expression of B7-H1 or B7-DC and clinicopathological variables or prognosis of patients, but no statistically significant relationship was found. We also examined expression of B7h, which is a ligand for ICOS, but no obvious expression in surgically resected specimens of non-small cell lung cancer was noted (data not shown). Expression of two other B7 family members, B7.1 and B7.2, is undetectable or low in most tumor cells (23–25). Thus, expression of B7 family members differs among tumor tissues.

Tumor cells evade host immune surveillance by several strategies. These include down-regulation of cell surface major histocompatibility complex class I molecules (24, 26), secretion of immunosuppressive factors [e.g., transforming growth factor β and IL-10 (27, 28)], lack of T-cell costimulation (24, 25, 29), and expression of death ligands or negative ligands (30, 31). Recently, B7-H1 has been shown to be involved in negative regulation of immune responses through the PD-1 receptor on activated T and B cells (8, 9) and has been thought to be a candidate strategy by which cancer cells evade host immune surveillance. The present study is the first to demonstrate in human tumor tissue that the number of TILs in B7-H1-positive tumor regions is significantly lower than that in B7-H1-negative tumor regions and, more importantly, that the number of PD-1-positive TILs in B7-H1-positive regions is significantly lower than that in B7-H1-negative regions. These findings suggest that B7-H1 on tumor cells might contribute to negative regulation against TILs in non-small cell lung cancer. B7-H1 expression on tumor cells may inhibit infiltration of PD-1-expressing TILs or cause down-regulation and apoptosis of infiltrated PD-1-expressing TILs.

The mechanisms regulating B7-H1 expression on tumor cells are not known. Inflammatory mediators are implicated by up-regulation of B7-H1 expression on the surface of several tumor cell lines after exposure to interferon γ (13, 16). Moreover, B7-H1 expression is more frequent in freshly isolated cancer tissue specimens than in cultured tumor cell lines (13), and the expression of B7-H1 on tumor-related dendritic cells may be up-regulated by tumor environmental factors (IL-10 or vascular endothelial growth factor) of ovarian cancer (32). These observations suggest that the cytokine microenvironment induces the expression of B7-H1 on tumor cells. On the other hand, T cells or natural killer cells that infiltrate tumor tissue secrete many cytokines, including interferon γ. Therefore, one possible scenario is that TILs secrete interferon γ in the beginning, followed by up-regulation of B7-H1 on tumor cells; thereafter, the up-regulated B7-H1 on tumor cells induces T-cell apoptosis via PD-1. Although the duration of B7-H1 expression on tumor cells after B7-H1 up-regulation is unclear, immunohistochemical staining might elucidate one step in these sequential events.

On the other hand, B7-H1 expression was absent in several cases in the present study, although TILs existed in most of these cases. This absence is most likely due to original tumor features. This finding is supported by a previous study, in which B7-H1 expression in several tumor cells was not detected even after exposure to interferon γ (17).

In contrast to the negative regulatory functions of B7-H1 and B7-DC against T-cell activation, positive costimulatory functions of B7-H1 and B7-DC in T-cell proliferation and cytokine production have been reported recently, and the functions of these molecules remain controversial. Dong et al. (5) reported that B7-H1 costimulated proliferative responses of T cells and induced IL-10 production on polyclonal T-cell stimulte and allogeneic antigens via IL-2-dependent process. Tseng et al. (33) reported that B7-DC costimulated T-cell proliferative responses and interferon γ production to greater levels than B7.1. Unlike B7-H1-expressing tumor cells, in one study, B7-DC-transfected tumor cells increased the number of antigen-specific T cells and were rapidly rejected in vivo by a PD-1-independent mechanism (34). Additional studies are needed to resolve the precise mechanisms by which B7-H1 and B7-DC affect tumor immunity. Moreover, it has been reported recently (13, 16) that tumor-associated B7-H1 negatively regulates T-cell activation through receptors that remain unidentified, with the exception of PD-1. Additional studies related to tumor-associated B7-H1 and its receptor in non-small cell lung cancer are also required.

In conclusion, we have demonstrated the expression of B7-H1 and B7-DC in surgically resected specimens of non-small cell lung cancer. Moreover, the number of TILs and PD-1 expression on TILs in B7-H1-positive tumor regions were significantly lower than that in negative regions. These results suggest that the expression of B7-H1 on tumor cells might contribute to negative regulatory immune responses against TILs in non-small cell lung cancer. Recently, it has been reported that B7-H1 blockade improves antitumor immunity and represents one approach for cancer immunotherapy (13, 17, 18, 32). The blockade of B7-H1 in non-small cell lung cancer might be one strategy to pursue for future immunotherapy in non-small cell lung cancer.

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