Blockade of B7-H1 or B7-DC induces an anti-tumor effect in a mouse pancreatic cancer model

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Abstract. The negative signal provided by interactions of programmed death-1 (PD-1) and its ligands, B7-H1 and B7-DC, has been suggested to play an important role in tumor evasion from host immunity. Pancreas cancer patients with B7-H1 expression have a poor prognosis. B7-H1 blocking has been shown to inhibit the development of a subcutaneous tumor from a pancreas cancer cell line. In this study, we investigated the effects of B7-DC as well as B7-H1 blockade in vivo in a murine pancreatic cancer model. Pancreatic cancer cells (Panc02) were inoculated in the pancreas of C57BL/6 mice. Five weeks later, tumor sizes were measured and the mice bearing appropriate size of tumors received the following treatments. Blocking antibodies against B7-H1 or B7-DC (200 μg) were administered 3 times/week for 3 weeks. Cells infiltrating the tumors were characterized by immunohistochemistry. Effects of antibodies on cytokine and FoxP3 expression were examined by quantitative RT-PCR. In vitro cultured Panc02 cells expressed B7-H1 upon IFN-γ stimulation. However, expression of B7-H1 and B7-DC was found mainly on CD45-positive infiltrating cells and rarely on cancer cells in vivo. Treatment with both antibodies significantly decreased tumor growth in vivo. B7-DC blockade decreased the levels of IL-10 and FoxP3, suggesting that regulatory systems are mainly inhibited at the tumor site. B7-H1 blockade increased the levels of IFN-γ and FoxP3. Collectively, blocking of B7-H1 or B7-DC efficiently induced regression of pre-established pancreatic cancers by up-regulating IFN-γ production and down-regulating IL-10 production at the tumor site.

Introduction

Despite advances in surgical as well as non-surgical treatment efforts, pancreatic cancer remains a tumor with a poor prognosis, and epochal treatments are desired. Besides anticancer drugs and molecule-targeting therapy, there are high expectations for immune therapy. T-cell activation is regulated by the interactions of costimulatory receptors on T cells with their ligands on antigen-presenting cells (APCs). The well-characterized ligands are the B7 family molecules, including B7-1 (CD80), B7-2 (CD86), B7-H2 (B7RP-1), B7-H1 (PD-L1), B7-DC (PD-L2), B7-H3 (B7RP-2) and B7-H4 (B7x). B7-1 and B7-2 engage ICOS, whereas B7-H1 and B7-DC were found to interact with programmed death-1 (PD-1) expressed on activated T cells and B cells (8-10). B7-H1 expression is also found in human cancers, including melanoma and carcinomas of the lung, ovary, colon, bladder, breast, cervix, liver, head, and glioblastoma (13-17), whereas B7-DC expression in human cancer is thought to be relatively rare.
beads with anti-CD3 mAb. Latchman et al reported that both PD-1/B7-H1 and PD-1/B7-DC signals inhibited T-cell proliferation by blocking cell cycle progression but not by increasing cell death (7).

Effects of PD-1/PD-1 ligand signals on tumor immunity have been also investigated. Iwai et al suggested that PD-1/B7-H1 interaction might promote tumor escape (21). Nomi et al reported that PD-1/B7-H1 interaction plays a negative role in tumor immunity in tumors from a pancreas cancer cell line, although tumors are located subcutaneously, but not inoculated in the pancreas itself (22). Dong et al (23) reported that B7-H1 was expressed on a large array of cancer cells and induced apoptosis of activated T cells. On the other hand, Shin et al reported that B7-DC signals significantly enhanced antitumor immunity (24). Liu et al suggested that B7-DC-related anti-tumor immunity might depend on a PD-1-independent mechanism (15). Although many researchers have suggested that PD-1/B7-H1 interaction regulates anti-tumor immunity (21,23), the role of PD-1/B7-DC interaction in tumor growth has not been clarified.

Recent studies have highlighted the significance of regulatory T cells (Treg cells) in the immune system (25,26). In terms of tumor immunity, Treg cells have been shown to suppress tumor-specific T-cell immunity and therefore may contribute to the progression of human tumors, including pancreatic cancer (27-29). In mice, depletion of Treg cells by Ab therapy leads to more efficient tumor rejection (30). Treg cell-mediated suppression of anti-tumor immune responses may partly explain the poor clinical response to vaccine-based immunotherapy for human cancer. B7-DC-positive DCs play an immunomodulatory role not only by secreting IL-10 but also by inducing proliferation of Treg cells (31).

We previously established a pancreas cancer model by inoculation of pancreas cancer cells into pancreas tissue, which is more appropriate for studying pancreas cancer immunology than using subcutaneous tumors (32). Since surface expression of adhesion molecules on inflammatory cells regulates organ-specific recruitment, cancer immunity against a subcutaneous tumor may differ from that of a tumor in the pancreas, where gut-tropic infiltrating cells mainly play a role. Using this model, we demonstrated that administration of recombinant CCL2 in tumor tissue induced significant tumor take ratio was 100% at 5 weeks after tumor inoculation, and the abdomen of each mouse was opened by a left flank incision and measured tumor sizes. Tumor take ratio was 100% at 5 weeks after orthotopic inoculation. Tumor volumes were approximated using the ellipsoidal formula: length x width x height x 0.52. Beforehand, small tumors with a diameter of less than 2 mm were excluded because volume calculation was inaccurate. In addition, large tumors with a diameter of more than 10 mm were also excluded because central necrosis was more or less found. Mice with peritoneal metastasis or cancerous ascites were also excluded.

Treatment protocol. We investigated the anti-tumor effects of the anti-B7-H1 antibody (MH5, rat IgG2a) and anti-B7-DC antibody (MH37, rat IgG2a) on pancreatic tumor. In some studies, mice received both anti-B7-H1 and anti-B7-DC antibodies. These antibodies were prepared as described previously (12,34). For injection of these antibodies, 200 μg antibodies were injected intraperitoneally by a 30 G needle. We injected these antibodies three times a week for three weeks. As controls, mice were sham-operated and the same amount of rat IgG (Beckman Coulter, Fullerton, CA) was applied in the same way for the same period. After the protocol was completed, we dissected these mice and measured tumor sizes. Tumor proliferation rate was calculated using the formula: volume on the last day/volume on the first day x (%).

Flow cytometry. Cell surface expression of costimulatory molecules on Panc02 cells in vitro was determined. Panc02 cells were treated in the presence or in the absence of IFN-γ (20 ng/ml) for 4 h before the cell preparation. For the determination of cell surface expression of costimulatory molecules on isolated tumor cells, sample was obtained from control pancreatic cancer tissue. The main bulk of tumor was minced into 1 to 3-mm3 chunks using sterile scalpel, and placed in sterile RPMI-1640 medium (Sigma, St. Louis, MO) containing collagenase (0.2%; Wako, Osaka, Japan) and DNAase (5 mg/ml; Roche, Indianapolis, IN). Tumor digestion was performed for 120 min at 37°C in a sterile bottle. Enzyme medium (10 ml) was added per 3 g of tumor chunks. Following the tumor digestion, the single-cell suspension was filtered through a tissue dissociator grid, and the resultant suspension was washed twice and submitted to immunofluorescent staining of cells for flow cytometry. For immunofluorescence staining, 1x10^6 cells in 25 μl medium were
incubated with anti-mouse CD16/32 (2,4G2, BD PharMingen, Franklin Lakes, NJ), followed by incubation with fluorescein isothiocyanate (FITC)-conjugated anti-mouse PD-1 antibody (J43, BD PharMingen), anti-mouse B7-H1 antibody (MIH5, eBioscience), anti-mouse B7-DC antibody (TY25, eBioscience), PE-conjugated anti-CD45 (13/2.3, Southern Biotech, Birmingham, AL), PE-conjugated anti-CD4 (GK1.5, Biolegend, San Diego, CA), PE-conjugated anti-CD8a (53-6.7, Biolegend), PE-conjugated anti-CD11b (M1/70, Biolegend), PE-conjugated anti-CD11c (N418, Biolegend), biotinylated anti-CD68 (MCA 1957B, Serotec, Oxford, UK) for 30 min on ice. PE-conjugated streptavidin (Phar Mingen) was incubated after anti-CD68 antibody reaction. Cells were analyzed on a FACS Calibur cytometer using CellQuest software (Becton Dickinson, San Jose, CA).

**Immunohistochemistry for analysis of infiltrating cells, co-stimulatory molecules and FoxP3.** The localization of CD4+, CD8+, CD11b+ and CD68+ cells was assessed by immunohistochemistry using the labeled streptavidin biotin method in control tumor tissue. Co-localization of CD4 and FoxP3 was assessed by fluorescent immunohistochemistry in control and antibody-treated tumors. Tumors were isolated and fixed in periodate, lysine-paraform-aldehyde solution, and tissues were embedded in an optimum cutting temperature compound (Takara Biomedicals, Osaka, Japan), 1 μg RNA and 0.2 μM tissue in the same way. RT-PCR was performed by using an ABI PRISM 9600 Sequence Detector (Applied Biosystems). Amplifications were generated by 10 min at 95˚C and then 40 cycles of denaturation at 95˚C for 15 sec, annealing, and extension at 62˚C for 1 min by using TaqMan universal PCR master mix kit and Assays-on-Demand Gene Expression probes (IFN-γ; Mm00801778, IL-10; Mm00439616_m1, FoxP3; Mm00475156_m1, Applied Biosystems). To standardize the quantitation of five selected genes, 18S RNA from each sample was quantified on the same plate with the target genes by using TaqMan 18S RNA Control Reagents kit (Applied Biosystems).

**Statistics.** All results are expressed as means ± SD of 5-8 mice. For comparison of tumor suppression rates, cell infiltration and cytokine expression, the mean values were statistically evaluated by Welch's t-test. Statistical significance was defined as p<0.05.

**Results**

**Expression of PD-1, B7-H1 and B7-DC on pancreatic cancer cells.** We first examined the expression of PD-1, B7-H1 and B7-DC on in vitro cultured Panc02 by flow cytometry. Fig. 1A showed that Panc02 cells weakly expressed PD-1, B7-H1 and B7-DC. However, the level of B7-H1 expression markedly increased after IFN-γ treatment. The expression of PD-1 slightly increased and the expression of B7-DC did not change. Next, we investigated the expression of these molecules in tumor tissue. Two-color flow cytometric analysis of isolated cells from tumor tissue showed that most of PD-1-positive cells, B7-H1-positive cells and B7-DC-positive cells was expressed on CD45+ cells, suggesting that they were expressed on infiltrating leukocytes (Fig. 1B). In addition, B7-H1 is also expressed in some CD45-negative cells, suggesting that cancer cells also expressed B7-H1 in accordance with the results of flow cytometry. In detail, some of CD4-positive cells expressed PD-1 and B7-H1 but not B7-DC. Most of CD8-positive cells also expressed PD-1. CD11b-positive cells expressed PD-1, CD11c-positive cells expressed B7-H1 and B7-DC. CD68-positive cells expressed PD-1 and B7-H1 and slightly expressed B7-DC. Taken together, these results suggested that PD-1 was mainly expressed on infiltrating CD4/8 T cells, B7-DC was expressed on infiltrating APCs and B7-H1 was expressed on both of them.

**Suppression of tumor growth by anti-B7-H1 and anti-B7-DC antibodies.** We examined the effects of blocking antibodies against B7-H1 and B7-DC on Panc02 tumor growth in vivo. Panc02 cells were directly injected into the pancreases and tumor volumes were measured 5 weeks later. We chose the mice which had appropriate sizes of cancer and treated with each antibody for three weeks. Fig. 2 shows the inhibitory effects of each antibody on the tumor growth after the three-week treatment. As compared with the sham-operated control group, each antibody had significant inhibitory effects. These results suggested that blocking of either B7-H1 or B7-DC
Figure 1. (A) Expression of PD-1, B7-H1 and B7-DC on Panc01 tumor cells in vitro (upper panel). Effect of IFN-γ treatment (20 ng/ml for 4 h) in vitro is shown in the lower panels. A marked increase of B7-H1 was observed. (B) Expression of PD-1, B7-H1 and B7-DC on tumor-infiltrating CD45, CD4, CD8, CD11b, CD11c and CD68. Representative flow data are demonstrated. Single-cells derived from tumor tissue were evaluated by flow cytometry. Expressions of PD-1, B7-H1 and B7-DC on the tumor infiltrating leukocytes are shown. Gating by forward/side scatter (FSC/SSC) was determined individually by single staining of CD45, CD4, CD8, CD11b, CD11c and CD68.
was efficient in inhibiting tumor growth. A combinational treatment with anti-B7-H1 and anti-B7-DC antibodies showed no additional effect compared with single treatment.

Effects on infiltration of CD4+, CD8+, CD11b+ and CD68+ cells in pancreatic cancer. We examined the infiltration of CD4+, CD8+, CD11b+ and CD68+ cells in tumor tissues on day 21 by immunohistochemistry (Fig. 3a and b). In sham-operated tumor tissues, small numbers of CD4+ cells were found. As compared with this, infiltration of CD4+ cells was significantly increased by either anti-B7-H1 or anti-B7-DC antibody treatment. Only a small number of CD8+ cell infiltrations were observed in control tumor tissue. Treatment with anti-B7-H1 or anti-B7-DC antibody resulted in a marked increase of CD8+ cell infiltration (Fig. 3a). CD11b+ and CD68+ cells were also detected in sham-operated tumor tissues. Infiltration of both CD11b+ and CD68+ cells were observed in whole tumor tissues, and the treatment with both antibodies (anti-B7-H1 and anti-B7-DC) enhanced the recruitment of these cells to a similar extent (Fig. 3b).

Effects on the expression of IFN-γ and IL-10 in pancreatic cancer. We next examined the expression of IFN-γ and IL-10 mRNA in tumor tissues by quantitative RT-PCR (Fig. 4). B7-H1 blockade induced a significant IFN-γ increase, suggesting that the anti-tumor effects of anti-B7-H1 antibody treatment might be mediated by IFN-γ. On the other hand, B7-DC blockade did not increase the expression of IFN-γ. In regard to IL-10 mRNA expression, the expression levels showed a different pattern from those of IFN-γ levels. In this case, B7-DC blockade remarkably decreased IL-10 mRNA expression, suggesting that the anti-tumor effect of B7-DC blockade might be due to the blocking of negative regulation by IL-10. In contrast, B7-H1 blockade did not show significant decrease in IL-10 mRNA expression.

Effects on infiltration of Treg cells in pancreatic cancer. As anti-tumor effect of anti-B7-DC treatment was related to decreased expression of IL-10, we speculated the participation of Treg cells in the regulation of tumor growth. Because B7-DC-positive DCs play an immunomodulatory role not only by secreting IL-10 but also by proliferating Treg cells (31). To prove this hypothesis, we analyzed the expression of forkhead box P3 (FoxP3) in tumor tissue. Treg cells are considered to suppress tumor-specific T-cell immunity and expression of FoxP3 is crucial for these cells. Fig. 5 shows the effects of these antibodies on the FoxP3 mRNA expression in tumor tissue. As well as IL-10 mRNA expression, anti-B7-DC antibody decreased FoxP3 mRNA expression, suggesting that participation of Treg cells in pancreatic tumor immunity and that blocking of this pathway might enhance anti-tumor immunity. On the other hand, anti-B7-H1 antibody did not decrease but rather increased FoxP3 expression. This suggests inhibition of Treg cells was not involved in the anti-tumor effects of anti-B7-H1 treatment. To confirm the FoxP3-expressing cells in tumors, dual-color immunohistochemistry was performed (Fig. 6). In control tumor tissue, FoxP3 was expressed in infiltrating CD4+ cells. In the anti-B7-H1-treated tumors, congestions of CD4+positive cells were observed and aggregates of CD4/FoxP3 double-positive cells were observed. In the anti-B7-DC-treated tumors, consistent with the mRNA expression analysis, FoxP3-positive cells were not observed.

Discussion
In this study, we demonstrated anti-tumor effects of B7-H1 or B7-DC blockade in vivo by using a murine pancreas cancer model. It is well known that PD-1/B7-H1/B7-DC interactions induce a negative regulation, which is critical for immune homeostasis after activation of T cells (2,7,10,18). This negative regulation is thought to be also beneficial for cancer cells to escape from tumor-specific T-cell immunity (21,23,35). There has also been a study using a pancreas cancer cell line that showed B7-H1 blocking inhibited tumor development, although interpretation of the results of this study is difficult because the tumors were subcutaneously injected (22). We used a pancreas cancer model established by local injection of cancer cells into the pancreas because cancer immunity is highly regulated by organ-specific leukocyte recruitment. As a result, blocking of B7-H1 inhibited tumor growth in our pancreas cancer model as in the above-described study, suggesting that B7-H1 is a possible target for treatment of pancreas cancer. We also investigated the role of B7-DC in our pancreas cancer model. The role of B7-DC has not been clearly characterized because expression of B7-DC on tumor cells is rare compared to expression of B7-H1 (15). In this study, PD-1 and B7-DC were mainly expressed on CD45-positive cells in tumor tissues, suggesting that they are expressed on tumor-infiltrating leukocytes. B7-H1 was expressed on CD45-negative cells as well as on CD45-positive cells, suggesting that pancreatic tumor cells express B7-H1. Freeman et al (2) reported that both B7-H1 and B7-DC reduced T-cell proliferation. In accordance with their findings, we found that the number of tumor-infiltrating cells was increased after either anti-B7-H1 or anti-B7-DC antibody treatment. In addition, expression of IFN-γ was also significantly increased after anti-B7-H1 antibody treatment. Increase in IFN-γ by blocking of the PD-1/B7-H1 pathway

Figure 2. Effect of anti-B7-H1 and anti-B7-DC antibodies on tumor growth. Tumor growth rate was calculated using the formula: (volume on the day 21/volume on the day 0)x100%. *p<0.01.
Figure 3. (a) Analysis of infiltrating CD4 or CD8 cells by immunohistochemistry. Sham-operated group (A, D and G), anti-B7-H1 treated group (B, E and H) and anti-B7-DC treated group (C, F and I). CD4 in lower magnification (A, B and C) and higher magnification (D, E and F); CD8 in higher magnification (G, H and I). Bars indicate 100 μm. (b) Analysis of infiltrating CD68 or CD11b cells by immunohistochemistry. Sham-operated group (A, D, G and J), anti-B7-H1-treated group (B, E, H and K) and anti-B7-DC-treated group (C, F, I and L). CD68 in lower magnification (A, B and C) and higher magnification (D, E and F); CD11b in lower magnification (G, H and I), CD11b in higher magnification (J, K and L). Bars indicate 100 μm.
has been demonstrated in several models, including chronic infectious diseases, in addition to cancer immunity (36).

Although treatment with anti-B7-H1 antibody failed to decrease FoxP3 expression, it increased the expression of IFN-γ. Because anti-B7-H1 treatment increased infiltration of CD8+ cells to tumor tissue, we speculated that IFN-γ was mainly produced by these infiltrating CD8+ cells. The increased IFN-γ in tumor tissue might contribute to the anti-tumor effect, because a large amount of IFN-γ expression from effector T cells for a long period can induce infiltration of inflammatory cells such as M1 macrophages which enhance anti-tumor immunity (21,37). Thus, it is conceivable that the suppressive effect of anti-B7H1 ligand antibodies on tumor growth can be mainly explained by the increased number of tumor-infiltrating effector cells. In other words, B7-H1 might attenuate tumor immunity in this cancer model by decreasing the infiltration of IFN-γ-producing T cells and M1 macrophages. Cells isolated from a pancreatic tumor expressed B7-H1 and Panc02 cells strongly expressed B7-H1 after IFN-γ treatment in vitro. Thus, B7-H1 induced on Panc02 tumor cells might also contribute to the evasion of tumor cells, which was abrogated by anti-B7-H1 treatment, leading to tumor growth inhibition.

Recent studies have highlighted the significance of Treg cells in the immune system (25,26). Treg cells have been shown to suppress tumor-specific T-cell immunity and therefore, may contribute to the progression of human cancers (27-29). Furthermore, tumor-infiltrating Treg cells are associated with reduced survival in patients with ovarian carcinoma (29). Therefore, we analyzed the expression of FoxP3, which is the master gene of Treg cells, to explore the participation of Treg cells in our pancreatic cancer model. We observed that B7-DC blockade achieved a significant decrease in FoxP3 expression in the tumor, although the number of tumor-infiltrating CD4+ T cells did not decrease after B7-DC blockade. Taken together, the results suggested that B7-DC blockade can decrease the number of infiltrating Treg cells. Furthermore, we quantified the expression of cytokines such as IL-10 and IFN-γ in the tumor tissue and observed a significant decrease in IL-10. This decrease in IL-10 might be at least partly related to the decrease in Treg cell infiltration. It is still controversial whether IL-10 is responsible for the Treg-mediated suppression of anti-tumor immunity. Dieckmann et al (38) suggested that suppression by Treg cells required direct cell contact between Treg cells and target T cells in in vitro experimental models. Furthermore, the results of a human study suggest that Treg cells must be activated through their TCR in order to be operationally suppressive (39). Membrane-bound TGF-β, CTLA-4 and other inhibitory co-stimulatory molecules, including B7-H1 and B7-DC, are thought to be critical for Treg cell-mediated suppression (40-43). Therefore, we speculate that the B7-DC blockade might regulate the suppressive effect of Treg cells on effector T cells by inhibiting the migration of Treg cells into tumors and thus direct contact with effector T cells. Alternatively, B7-DC might be involved in expansion of Treg cells (31).
We propose that this murine pancreatic cancer model escaped from anti-tumor immunity by two independent mechanisms: decreasing infiltration of IFN-γ-producing effector T cells by B7-H1 and increasing Treg cells by B7-DC. Thus, we studied the combination of anti-B7-H1 and anti-B7-DC antibodies in expectation of a stronger inhibitory effect than that of single treatment. However, the combination therapy unexpectedly did not show an additive anti-tumor effect. Messenger RNA expression profile in the tumor showed that the combination therapy did not decrease IL-10 or FoxP3, while it successfully increased IFN-γ, like single treatment with anti-B7-H1 (data not shown). This indicates a dominant effect of anti-B7-H1 over that of anti-B7-DC and further implies differential roles of B7-H1 and B7-DC in regulating anti-tumor immunity.

In summary, we achieved significant tumor suppression by blocking B7-H1 or B7-DC. Blockade of B7-H1 increased intra-tumoral IFN-γ level and infiltration of inflammatory macrophages, which might directly lead to the anti-tumor effect. In contrast, B7-DC blockade decreased tumor infiltration of Treg cells and intra-tumoral IL-10, which might be primarily responsible for the anti-tumor effect. These differential roles of B7-H1 and B7-DC in tumor immune evasion may be a good target for clinical treatments of pancreatic cancers.

References


