Blockade of the Interaction Between PD-1 and PD-L1 Accelerates Graft Arterial Disease in Cardiac Allografts

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Background—Programmed death-1 (PD-1), a member of the CD28 family, has been identified. PD-1 is involved in the negative regulation of some immune responses. We evaluated the role of PD-ligand 1 (PD-L1) in graft arterial disease (GAD) of cardiac allografts and in smooth muscle cells (SMCs).

Methods and Results—C57BL/6 murine hearts were transplanted into B6.C-H2<bm12>KhEg mice for examination of GAD. PD-L1 was expressed in SMCs of the thickened intima in the graft coronary arteries, and administration of anti–PD-L1 monoclonal antibody (mAb) enhanced the progression of GAD (luminal occlusion: 55±5.0% versus 9.8±4.3%, *P*<0.05). The expressions of interferon γ (IFN-γ) and tumor necrosis factor α of cardiac allografts were upregulated in response to anti–PD-L1 mAb treatment. In vitro, PD-L1 expression was induced in SMCs in response to IFN-γ stimulation. Sensitized splenocytes increased SMC proliferation, and anti–PD-L1 mAb in combination with IFN-γ stimulation increased this proliferation.

Conclusions—The PD-L1 pathway regulates both the proliferation of SMCs and GAD. Thus, control of this interaction is a promising strategy for suppression of GAD. (Arterioscler Thromb Vasc Biol. 2004;24:2057-2062.)

Key Words: transplantation ▪ graft arterial disease ▪ smooth muscle cell ▪ immune system ▪ B7 family

Cardiac allograft transplantation developed as a therapy for end-stage congestive heart failure. The survival rate at 1 year after transplantation has increased to >80% by introduction of immunosuppressive drugs. However, the use of the drugs also results in opportunistic infection, and these drugs do not prevent graft arterial disease (GAD), which occurs in chronic rejection.

Programmed death-1 (PD-1) is a member of the CD28 family that was identified in a T cell line undergoing programmed cell death, but subsequent studies have shown that its expression is associated with lymphocyte activation rather than cell death. PD-1 contains an immunoreceptor tyrosine-based inhibitory motif in its cytoplasmic tail. C57BL/6 mice lacking PD-1 develop lupus-like arthritis and glomerulonephritis, and Balb/c mice lacking PD-1 develop fatal dilated cardiomyopathy. Okazaki et al purified the 30-kDa protein from heart extract and identified it as cardiac troponin I in these dilated cardiomyopathy mice, and administration of anti–cardiac troponin I (anti-cTnI) antibody induced heart dilation and dysfunction in wild-type mice. The data suggest that PD-1 receptor engagement leads to downregulation of immune responses. Furthermore, PD-ligand 1 (PD-L1) was recently identified. PD-1 ligand shows a tissue distribution profile distinct from that of the other B7 family members. Expression of PD-L1 is upregulated on activation of antigen presenting cells, including dendritic cells, monocytes, and B cells. In addition, PD-L1 has been detected in lymphoid as well as in nonlymphoid organs.

Although Ozkaynak et al reported the expressions and functional roles of PD-1 and PD-L1 in the pathogenesis of cardiac transplants, the relation between the PD-1/PD-L1 pathway and the proliferation of smooth muscle cells (SMCs) has not been investigated. In the present study, we examined the expression of PD-1 and PD-L1 in relation to chronic rejection in murine heterotopic cardiac transplantation models and investigated the effect of monoclonal antibody (mAb) against PD-L1 in these models.

Methods

Reagents

Anti-mouse PD-L1 (MIH5, rat immunoglobulin G2a [IgG2a]) mAb was generated as described previously. Anti-mouse PD-1 mAb, fluorescein isothiocyanate (FITC)-conjugated anti-mouse α-smooth muscle actin (αSMA) mAb, anti-mouse CD4 mAb, FITC-conjugated anti-mouse CD4 mAb, anti-mouse CD8α mAb, FITC-conjugated anti-mouse CD8α mAb, FITC-conjugated anti-mouse CD11b mAb, FITC-conjugated anti-mouse CD11c mAb, streptavidin-phycocerythrin, streptavidin-Texas red, anti-mouse interferon γ (IFN-γ; XMG1.2), anti-mouse tumor necrosis factor α (TNF-α), and recombinant mouse IFN-γ were purchased from BD PharMingen (San Diego, Calif). Nonneutralizing anti-mouse CD18 mAb (1B/2, rat IgG2a) was purchased from the American...
Type Culture Collection (Rockville, Md). Anti-actin antibody was purchased from CHEMICON International (Temecula, Calif). Biotinylated isotype-matched antibodies were used as controls.

Cardiac Transplantation and Treatment
Male C57BL/6 (B6; H-2b) mice aged 6 to 8 weeks were obtained from Japan Crea Laboratories (Tokyo, Japan). B6.C-H2-<bm12>KhEg (Bm12; H-2bm12) mice were obtained from The Jackson Laboratories (Bar Harbor, Me). Mice were housed in a specific pathogen-free mouse facility. Donor hearts were transplanted heterotopically into the recipient’s abdomen. B6 mice were used as donors, and Bm12 mice were used as recipients, for assessment of GAD as described previously (class II mismatch). Anti–PD-L1 mAb and control IgG were suspended in PBS solution. Recipient mice were injected intraperitoneally with anti–PD-L1 mAb (100 or 200 µg/d) or control IgG (200 µg/d) twice a week for 4 weeks.

This study conformed to the Guide for the Care and Use of the Laboratory Animals of Tokyo Medical and Dental University and was approved by the internal committee.

Graft Harvest
Donor hearts were harvested 8 weeks after transplantation to assess the expression of PD-L1 in GAD or were harvested 4 weeks after transplantation to assess the effect of blockade of the interaction between PD-1 and PD-L1. Harvested heart allografts were sectioned transversely into 3 parts: the basal section was fixed with 10% phosphate-buffered formalin and embedded in paraffin for morphological examination, the midportion was frozen in OCT compound and stored at −80°C for immunohistochemical staining, and the apical portion was used for Western blotting.

Histological Evaluation
For assessment of GAD, grafts were analyzed in sections stained with Elastica van Gieson to show the internal elastic lamina. The percentage of the occluded lumen was calculated by means of an image analysis system (Scion Image) as described previously.

Immunohistochemistry and Confocal Laser Scanning Microscopy
Frozen sections (5 µm) were incubated with 10% normal rabbit serum at room temperature. Sections were then incubated with anti–CD4 or anti–CD8 antibody overnight at 4°C. After incubation with secondary antibody (biotinylated rabbit anti-rat IgG), antigen-antibody conjugates were detected with avidin-biotin-horseradish peroxidase complex (Nichirei, Tokyo, Japan) according to the manufacturer’s instructions. Five hearts from each group were examined, and the CD4 or CD8 positive cells per high-power field were counted in 5 random fields; the counts were then averaged.

PD-1 or PD-L1 expression was visualized with a Tyramide Signal Amplification Biotin System (MEN Life Sciences) as described previously. Briefly, sections were incubated in biotinylated rabbit anti-rat IgG, antigen-antibody conjugates were detected with avidin-biotin-horseradish peroxidase complex (Nichirei, Tokyo, Japan) according to the manufacturer’s instructions. Five hearts from each group were examined, and the CD4 or CD8 positive cells per high-power field were counted in 5 random fields; the counts were then averaged.

Statistical Analysis

All data are expressed as mean±SEM. Data were compared, and differences between 2 groups were analyzed by Student t test. Differences in data between multiple groups were subjected to ANOVA followed by Scheffé test. Differences were considered statistically significant at a value of P<0.05.

Results

Expression of PD-1 and PD-L1 in Cardiac Allografts and Splenocytes
Cardiac allografts showed cell infiltration, and some of these cells expressed PD-1 (Figure 1A) and PD-L1 (Figure 1B). Neither PD-1 nor PD-L1 was expressed in native hearts (Figure 1A and IB, available online at http://atvb.ahajournals.org). As detected by flow cytometry, PD-L1 was expressed in CD8-positive splenocytes from Bm12 mice before transplantation (Figure II A, available online at http://atvb.ahajournals.org). Furthermore, most CD4-, CD11b-, and CD11c-positive splenocytes obtained from mice at 2 weeks after transplantation expressed PD-L1 (Figure II B). PD-L1 was not expressed in splenocytes from Bm12 mice before transplantation (Figure II A), but it was

Flow Cytometry
Splenocytes were isolated from recipients as previously described. Cells were stained with biotinylated anti–PD-1 mAb, anti–PD-L1 mAb, or isotype-matched control IgG. Cells were then stained with FITC-conjugated anti–CD4, anti–CD8, anti–CD11b, or anti–CD11c antibody and streptavidin-phycocerythrin. Cultured SMCs were stained with biotinylated anti–PD-1 mAb, anti–PD-L1 mAb, or isotype-matched control IgG, and then stained with FITC-conjugated anti–αSMA antibody. Flow cytometry was performed with a FACS Calibur and Cell Quest software (BD Biosciences).

Western Blot Analysis
Snap-frozen tissues from cardiac allografts and native hearts were extracted in 1 mL of solution containing 50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1% Triton X-100, 2 mmol/L EGTA, 10 mmol/L EDTA, 100 mmol/L NaF, 1 mmol/L Na3P2O4, 2 mmol/L Na2VO4, 100 µg/mL phenylmethylsulfonyl fluoride, and cocktail tablets (Roche, Germany) and homogenized. Homogenates were centrifuged, and the supernatants were stored. Proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and incubated with primary antibody at 4°C overnight. The membranes were incubated with secondary antibody (Amersham Biosciences, Piscataway, NJ) for 2 hours and developed with enhanced chemiluminescence reagent (Amersham Biosciences). Enhanced chemiluminescence was detected with an LAS-1000 (FujiFilm).
induced in splenocytes from recipient mice at 2 weeks after transplantation (Figure IIB).

Expression of PD-L1 in SMCs
Confocal microscopy showed that PD-L1 expression was induced in SMCs by 48 hours of stimulation with IFN-\(\gamma\)/H9253 (Figure III, available online at http://atvb.ahajournals.org). FACS analysis showed that PD-L1 was not expressed in nonstimulated SMCs, but stimulation with IFN-\(\gamma\)/H9253 for 24 hours induced PD-L1 expression. PD-1 was not expressed in nonstimulated or stimulated SMCs (Figure 2). We also confirmed mRNA of PD-1 and PD-L1 by RT-PCR, which showed PD-1 was not induced, but PD-L1 was induced in SMCs in response to IFN-\(\gamma\) (data not shown).

Expression of PD-L1 in GAD
Allografts at 8 weeks showed severe GAD (Figure 3A). Although PD-L1 was not expressed in coronary arteries of native hearts (Figure 3B), immunohistochemical examination showed expression of PD-L1 in coronary arteries of cardiac allografts after 4 weeks (Figure 3C). Immunohistochemical examination and confocal microscopy showed expression of PD-L1 in proliferated SMCs in GAD (Figure 3D and 3E).

Effect of Anti–PD-L1 mAb on GAD
During the observation period in our experiments, all allografts kept beating. There was no functional change in the grafts from mice treated with anti–PD-L1 mAb. We harvested allografts at 4 weeks after transplantation to test the effect of anti–PD-L1 mAb treatment. We examined the extent of myocardial lesion and that of vascular lesion as previously described\(^{19}\) to assess the effect of anti–PD-L1 mAb treatment. On histological examination, allografts in the 200 \(\mu\)g/d anti–PD-L1 mAb-treated group (n=6) showed severe GAD in comparison to allografts in the 100 \(\mu\)g/d–treated group (n=6) or control IgG group (n=6; luminal occlusion: 55±5.0% versus 21±5.6% versus 9.8±4.3%, respectively, \(P<0.05\); Figure 4A through 4C; Figure IVA, available online at http://atvb.ahajournals.org). The cellular infiltrate was examined immunohistochemically in allografts at 4 weeks after transplantation. The number of infiltrating CD4\(^+\) (Figure 4D through 4F) and CD8\(^+\) (Figure 4G through 4I) T cells in the anti–PD-L1 mAb-treated group was significantly increased compared with the control IgG group (Figure IVB and IVC). Next, we examined the perivascular fibrosis by Masson’s trichrome staining. In our models, there were so many infiltrating cells around coronary arteries (data not shown) that we could not identify the area of the perivascular

Figure 1. A and B, PD-1 and PD-L1 expression in cardiac allograft at 4 weeks. Representative microphotographs of immunohistochemical staining of PD-1 (A) or PD-L1 (B) are shown. Original magnification, \(\times400\).

Figure 2. PD-L1 expression in SMCs in response to IFN-\(\gamma\). FACS analysis of SMCs stimulated with IFN-\(\gamma\) (10 ng/mL) for 24, 48, and 72 hours is shown. Gray represents PD-1 or PD-L1, and the solid line represents isotype-control IgG.

Figure 3. A, Elastica van Gieson staining of cardiac allografts at 8 weeks after transplantation (original magnification, \(\times400\)). B through D, PD-L1 expression in coronary arteries of native heart and cardiac allografts (original magnification, \(\times400\)). B shows native heart; C, allografts at 4 weeks; and D, allografts at 8 weeks. E, Confocal microscopy of GAD at 8 weeks. (Green shows \(\alpha\)-SMA; Red, PD-L1; original magnification, \(\times400\)).
fibrosis; therefore, we could not analyze quantitative difference in the extent of the perivascular fibrosis between groups.

**Effect of Anti–PD-L1 mAb on Cytokine Expression**

Western blot analysis showed that the expressions of IFN-γ and TNF-α were upregulated in the 200 µg/d anti–PD-L1 mAb-treated group compared with those in the control IgG group or in native hearts (Figure 5). Although we also examined the expression of adhesion molecules, the expressions of intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 were not changed (data not shown).

**Discussion**

GAD involves an allogeneic immune response, and it likely develops from intimal perivascular recruitment and activation of macrophages and T cells. Recognition of foreign tissue by T cell interaction with donor major histocompatibility complex promotes clonal expansion of the T cells; activated T cells then secrete a variety of cytokines including IFN-γ. These factors ultimately drive the SMC recruitment that forms the intimal hyperplastic lesion of GAD. Furthermore, costimulatory molecules are involved in the development of GAD. Treatment with CTLA4 Ig suppressed GAD in a rat transplantation model, and blockade of both inducible costimulator and the CTLA4 pathway suppressed GAD in a murine transplantation model. PD-L1, which belongs to the B7 family, is also induced in endothelial cells and SMCs in...
response to IFN-γ. However, there are only a few reports on the role of the PD-1/PD-L1 pathway in arteriosclerosis or other arterial lesions. In this study, the role of the PD-1/PD-L1 pathway in GAD and SMC proliferation was investigated in cardiac transplantation and in cultured SMCs.

The engagement of PD-1 by PD-L1 inhibits T cell receptor-mediated T cell proliferation and cytokine secretion by activated T cells but does not inhibit them when PD-1-deficient T cells are used in vitro. These findings indicate that the inhibitory signal for T cell activation is transduced by PD-1, and the PD-1/PD-L1 pathway may play an important role in suppression of excessive immune response. Indeed, in several autoimmune animal models including experimental autoimmune encephalomyelitis, autoimmune diabetes in prediabetic female nonobese diabetic mice, and murine hapten-induced contact hypersensitivity, blockade of the PD-1/PD-L1 pathway accelerates the time course and severity of the disease. Therefore, we considered that PD-L1 may play an important role in the suppression of GAD, and we hypothesized that blockade of the PD-1/PD-L1 pathway would lead to exacerbation of GAD, as is the case in other autoimmune animal models.

Some investigators reported, however, that PD-L1 co-stimulates T cell proliferation and cytokine production in vitro. Ozkaynak et al reported that administration of PD-L1–Ig fusion protein alone or coadministration with cyclosporine A prolonged cardiac allograft survival and suppressed GAD in CD28-deficient mice. Their data appear to be contrary to ours. There are 2 factors that could account for this discrepancy. First, the action of the Ig fusion protein was not examined in the Ozkaynak et al study. It is unclear whether PD-L1 co-stimulation is PD-1-dependent or whether it could be mediated by an alternative receptor for PD-L1. Some investigators assume the existence of a second receptor for PD-L1, although it has not been identified. Therefore, it is possible that PD-L1 Ig interacts with a second receptor. Second, the results of Ozkaynak et al are based on complex conditions, such as the absence of CD28 signals, during treatment with a calcineurin blocker. These points could explain the difference between our data and the Ozkaynak et al study.

The role of the PD-1/PD-L1 pathway in vivo remains controversial. Our study showed that blockade of the PD-1/PD-L1 pathway enhanced GAD and upregulated IFN-γ and TNF-α. Furthermore, SMC proliferation was increased in response to IFN-γ in vitro. IFN-γ is a key regulatory cytokine associated with chronic allograft rejection. IFN-γ is upregulated in rejected cardiac allografts, and it plays a critical role in the pathogenesis of coronary arteriosclerosis in transplanted hearts. Thus, our results indicate that the progression of GAD is mediated by the increased SMC proliferation in response to stimulation by IFN-γ. There are several reports on the relation between IFN-γ and SMC proliferation. Some showed that IFN-γ inhibited SMC proliferation, and others showed IFN-γ increased SMC proliferation. Under our conditions, the addition of IFN-γ increased SMC proliferation, and anti–IFN-γ mAb inhibited this proliferation. In consideration of the previous report that GAD was suppressed in IFN-γ-deficient mice, it is necessary to clarify the role of IFN-γ in SMC proliferation in detail. In accordance with our previous observation that TNF receptor-1 and -2 double deficiency reduces GAD, we showed that the treatment with anti–PD-L1 mAb increased the expression of TNF-α in this study. The upregulation of TNF-α was involved in the progression of GAD in the anti–PD-L1 mAb-treated mice.

PD-L1 was expressed in proliferated SMCs in GAD and in cultured SMCs stimulated by IFN-γ. PD-1 was not expressed in stimulated SMCs but was expressed in splenocytes stimulated by in vivo allograft transplantation. Thus, we hypothesized that treatment with anti–PD-L1 mAb has an effect not only on the interaction between T cells and dendritic cells but also on the interaction between T cells and SMCs. We showed that SMC proliferation was increased by stimulation with IFN-γ. In addition, blockade of the PD-1/PD-L1 pathway showed further increases in response to sensitized splenocytes. In our system, as splenocytes were inactivated, this increase was due to the SMC proliferation. Our investigation into the pathogenesis of GAD indicates that direct interaction between activated splenocytes and SMCs is critical in the process of intimal hyperplasia. This is in addition to the direct effect of IFN-γ, a key regulatory cytokine, on SMC proliferation. We showed that PD-L1 is a regulatory molecule in SMC proliferation in response to alloantigen-stimulated splenocytes. However, nontreated cardiac allografts showed severe GAD after 8 weeks despite the expression of PD-L1. Thus, the regulation of the only PD-1/PD-L1 pathway is not sufficient to suppress SMC proliferation in vivo. We need to clarify the mechanisms by which PD-1/PD-L1 pathway and other costimulatory pathways work together to activate SMCs.

Our study has shown the association of the PD-1/PD-L1 pathway with SMC proliferation in vitro. There is increased evidence that inflammation and T cell immunity are involved in a variety of vascular lesions, particularly those in arteriosclerosis and atherosclerosis. Although the data in this study were limited to arterial lesions in relation to allograft rejection, it is reasonable to think that the PD-1/PD-L1 pathway could be involved in the pathophysiology of other vascular lesions including restenosis after balloon angioplasty.

We showed that PD-L1 is involved in the regulation of chronic rejection of cardiac allografts. Further studies are needed to clarify the differential roles of this and other pathways. Regulation of these molecules could provide a new strategy for the suppression of GAD in clinical heart transplantation.

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Figure I

A    PD-1
B    PD-L1
Figure I
PD-1 and PD-L1 expression in native hearts. Representative microphotographs of immunohistochemical staining of PD-1 (A) or PD-L1 (B). (original magnification, 400x).
Figure II A

- PD-1 → CD4
  - 0.7 0.8
  - 36.2
- PD-L1 → CD4
  - 0.6 1.6
  - 40.7
- Control IgG → CD4
  - 0.2 1.4
  - 44.9

- PD-1 → CD8
  - 1.5 0.1
  - 15.1
- PD-L1 → CD8
  - 53.0 17.6
  - 1.1
- Control IgG → CD8
  - 0.1 0.0
  - 18.1

- PD-1 → CD11b
  - 0.2 0.1
  - 6.5
- PD-L1 → CD11b
  - 18.9 1.0
  - 5.7
- Control IgG → CD11b
  - 0.0 0.0
  - 6.3

- PD-1 → CD11c
  - 0.4 0.2
  - 1.3
- PD-L1 → CD11c
  - 59.0 1.4
  - 0.4
- Control IgG → CD11c
  - 0.5 0.1
  - 1.9
Figure II B
Figure II

FACS analysis of splenocytes. (A) PD-1 and PD-L1 expression in splenocytes from normal Bm12 mice. PD-1 was not expressed in splenocytes from Bm12 mice before transplantation. (B) PD-1 and PD-L1 expression in splenocytes from recipient Bm12 mice at 2 weeks after transplantation. PD-1 was expressed in splenocytes from Bm12 after transplantation.
Figure III

(α-SMA) (PD-L1) (Merged)
Figure III
Confocal microscopy of SMCs. (Green: α-smooth muscle actin (αSMA), Red: PD-L1, original magnification, 400x). SMCs were stimulated with IFN-γ for 24 hours. PD-L1 was induced in SMCs.
Figure IV

A

Stenosis Rate (%)

Control IgG
Anti-PD-L1 (100)
Anti-PD-L1 (200)

B

Cells/HPF

Control IgG
Anti-PD-L1 (100)
Anti-PD-L1 (200)

C

Cells/HPF

Control IgG
Anti-PD-L1 (100)
Anti-PD-L1 (200)
Figure IV

(A) Quantitative analysis of luminal occlusion of coronary arteries. (*$P < 0.05$). (B and C) The number of CD4$^+$ (B) or CD8$^+$ (C) cells in control IgG group, anti-PD-L1 mAb-treated group (100 µg) or anti-PD-L1 mAb treated group (200 µg). (*$P < 0.05$).
Figure V

A

(IFN-γ (ng/ml))

B

(Anti-IFN-γ mAb (µg/ml))

C

(Anti-PD-L1 mAb (µg/ml))

D

(Anti-PD-L1 mAb (µg/ml))

E

(Anti-PD-L1 mAb (µg/ml))
Figure V

Proliferation of smooth muscle cells (SMCs). (A) The effect of IFN-γ on the proliferation of SMCs (*P < 0.05). (B) The effect of anti-IFN-γ mAb on SMCs proliferation. (*P < 0.05). (C) The effect of anti-PD-L1 mAb on SMCs in response to sensitized splenocytes without IFN-γ pretreatment. (MTT assay, *P < 0.05). (D) The effect of anti-PD-L1 mAb without splenocytes on SMC proliferation (MTT assay). (E) The effect of anti-PD-L1 mAb with anti-IFN-γ mAb on SMC proliferation in response to sensitized splenocytes. (*P < 0.05).