ORIGINAL ARTICLE

Function of PD-L1 in antitumor immunity of glioma cells

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Abstract Human glioma is a highly fatal tumor with a significant feature of immune suppression. The functions of PD-L1 refer to co-simulation and immune regulation. To investigate expression and functional activity of PD-L1 in human glioma cell in vivo and in vitro. Expressions of PD-L1mRNA and protein in the human glioma cell line were analyzed with quantitative RT-PCR and flow cytometer; and then expression of PD-L1 in tissue specimens of 10 glioma patients was treated with immunohistochemical analysis; glioma cell and allogeneic CD4+ and CD8+ T cells were co-cultured, and cytokine IFN-γ, IL-2 and IL-10 in cultured supernatant fluid were determined with ELISA; upon blocking the interaction between glioma cell and the immune cell with PD-L1 monoclonal antibody (5H1), surface markers on immune cells were analyzed using flow cytometer. All human glioma cell lines constitutively expressed PD-L1, and IFN-γ induced glioma cell to highly express PD-L1. It was shown through immunohistochemical analysis that glioma specimen expressed PD-L1, while expression of PD-L1 was not observed in normal tissue and normal human brain near the tumor location. The release of IFN-γ and IL-2 was inhibited, while IL-10 was increased slightly. Glioma cell may escape from immune recognition and injury with the help of PD-L1, which is a significant pathogenic mechanism of glioma.

1. Introduction

Glioma is known as glioblastoma and also neuroepithelial tumors or neuroectodermal tumors for it occurs in neuroectodermal regions. More and more evidence shows that the body produces tumor-specific T cell-mediated immunity. However, tumor-specific immune response is not sufficient to eradicate the neoplasm. Antigen-induced T cell activation and proliferation is regulated by the positive and negative costimulatory receptor of the immunoglobulin superfamily (Mavilio and
Glioma is a highly lethal cancer, and it is also a typical tumor that can inhibit the effective anti-tumor immune response (Walker et al., 2002; Ashraf et al., 2013). However, its pathogenesis remains unclear.

PD-L1, the homolog of B7.1/2 (CD80/86), shows the ability to co-stimulate molecules and regulate the immune system (Joshua et al., 2013; Butte et al., 2007). It is expressed in APCs and induced in the lymphoid and non-lymphoid peripheral tissues to make the outer periphery of PD-L1 for the regulation of activated T cells. IFN-γ is an effective cytokine for up-regulation of PD-L1, as IFN-γ response element in the promoter region of PD-L1. The expression of B7-DC (PD-L, another PD-1 ligand) is substantially confined to the medullary DCs and macrophages, not in peripheral tissues or wide expression in many cell types. Therefore the effect of inhibition of peripheral T cell-mediated immunity is less.

The immune system inspects and removes mutative cells, which are immune surveillance functions. Accordingly, the tumor cells may change phenotype to escape the attack of the immune response. Studies have shown that manipulation of the tumor microenvironment PD-L1/PD-1 molecule pathway and inducing expression of PD-L1 are associated with the immune suppression of the tumor, thereby allowing tumor progression and metastasis. PD-L1/PD-1 molecular pathway is the main mechanism of tumor immune escape, for the following reasons: First and most importantly, this approach is involved in the immune response, especially in the negative regulation of tumor origin and growth of the peripheral tissues; Secondly, the up-regulation of B7-H1 in the tumor microenvironment has also up-regulated the PD-1 in activated tumor infiltrating T cells, which may start a vicious suppressive loop; third, the pathway through a two-way signal interleave contact innate and acquired immune regulation. These factors make the PD-1/B7-H1 molecular pathways that control the immune response in cancer and its progression play a central role. That is, the molecular axis of PD-L1/PD-1 is controlled by the tumor led “good genes” to worse (Pardoll, 2012; Butte et al., 2015; Batoof et al., 2015).

In the present study, we show that the outer glioma cells in vivo have a high expression of PD-L1 and PD-L1 expressed by glioma cells reduces its immunogenicity in vitro. Thus, PD-L1 is a new mediator with an immunosuppressive effect in human gliomas.

2. Materials and methods

(1) Cell culture. Human malignant glioma cell lines LN-229, U251MG and LN-308 were purchased from the Wuhan University typical species preservation centers, these cells are cultured according to the literature methods (Wiendl et al., 2002).

(2) Immunohistochemistry. Tumor specimens including 9 glioblastoma (WHO grade IV) and a mixed glioma (WHO III, female, n = 6, age range, 30–82 years old; males n = 4, age range 60–71 years old) were obtained from the operation of inpatient neurosurgery of our hospital. A normal brain tissue and normal brain tissue adjacent to tumor cells were used as control tissue. Immunohistochemistry was in accordance with the literature (Wiendl et al., 2002). Frozen tissues were cut (20 μm) and fixed in acetone, using PD-L1 antibody (5H1) or isotype control antibody staining. The reaction product was observed with streptavidin-biotin staining (Shenzhen Nano Biotechnology Co., Ltd.). The percentages of positive staining of glioma cells as <25%, 25–50%, 50–75%, and >75% were quantitated.

(3) PBMCs. Purified lymphocytes and DCs. PBMCs were isolated from the peripheral blood of normal healthy people (Wiendl et al., 2002). Separated by MACS CD4⁺ and CD8⁺ T cells (Miltenyi Biotec, Bergisch Gladbach, Germany). Co-culture experiments were in accordance with the literature (Wiendl et al., 2002). Using ELISA (BD PharMingen) to detect cytokines that released into the supernatant (human IFN-γ, IL-2 and IL-10). The surface molecular markers of activated T cells were analyzed by flow cytometry. Mononuclear cells were obtained in RPMI1640 containing 10% fetal bovine serum with 1 h adhesion at 37 °C and cytokine GM-CSF (100 ng/ml, Leukomax; Sandoz, Basel, Switzerland) and IL-4 (40 ng/ml; PепроTech, Inc., Offenbach, Germany), after 6 days of culture these cells display an immature DC phenotype (CD14 and CD1a⁺ and HLA-DRlow, CD86low, CD80low and CD83). Maturation was induced by LPS (5 μg/ml, S. typhi; Sigma L-7261) or TNF-α (PепроTech EC Ltd 200 units/ml). Mature DC HLA-DR expression and co-stimulatory molecules (CD86 and CD80).

(4) Flow cytometry analysis. The immune cell surface markers were analyzed by CellQuest software (FACS Calibur cytometer; Becton Dickinson, Heidelberg, Germany). Count 10,000 events/antigen. The histogram was obtained by calculating SFI.

(5) Co-culture experiments. Co-culture experiments were performed according to the literature (Wiendl et al., 2003). 5 × 10⁵ glioma cells/well in 48-well culture plate types (Costar, Bodenheim, Germany), cultured with or without containing IFN-γ (500 units/ml) for 24 h to induce the expression of MHC-II PD-L1. The cells were washed with PBS, and 0.5 * 10⁶ freshly purified T cell types (CD4⁺ and CD8⁺) were added to them. Then the cells were co-cultured in RPMI1640. To describe glioma – correlation functions of immune cell interactions anti PD-L1 mAb (5H1) or a control antibody was added in accordance with the respective predetermined time or design. For all blocking experiments, co-culture was added in anti-PD-L1 (5H1). To detect the production of cytokines (human IFN-γ, IL-2 and IL-10), the supernatant was collected and analyzed by ELISA (BD PharMingen).

(6) RNA extraction, cDNA synthesis and quantitative RT-PCR. Total RNA was extracted using Trizol (Invitrogen) according to the reagents operating instructions. RevertAid First Strand cDNA Synthesis Kit (MBI Fermentas, St. Leon-Rot, Germany) was used for cDNA synthesis and PCR amplification and DyNAamo Flash SYBR Green qPCR kit (Finzymes Oy, Espoo, Finland) for Real-time PCR. Expression levels were calculated and normalized 18s rRNA. Primers 18s rRNA and PD-L1 are shown in Table 1.

(7) Statistical analysis. Data processing using SPSS 11.0 software package, data were described in ± sE and t-test was used to compare the differences between two groups. P < 0.05 indicates significant difference.
3. Results

The expression of PD-L1 in human glioma cell lines. Expressed by the following by to detect. The PD-L mRNA expression of three kinds of human glioma cell lines was detected by qRT-PCR with or without the presence of IFN-γ (500 units/ml, 48 h). All glioma cell lines consisted of low-expressing PD-L1 mRNA. In the presence of IFN-γ, PD-L1 transcription (LN-308) increased 4.2-fold (Fig. 1A). Compared with IFN-γ, TNF-α has no effects on the expression of PD-L1 mRNA (data not shown). Flow cytometric analysis of glioma cell lines revealed that the entire surface thereof has a composition formula of PD-L1 expression. After stimulation with IFN-γ, PD-L1 expression enhanced from 3.6 times to 17.2 times with SFI ratio (LN-229) (Fig. 1B).

1. PD-L1 expression of Gliomas in vivo. By immunohistochemical staining, PD-L1 expressed in nine glioblastoma (WHOIV) and a mixed glioma. More than 50% of the tumor cells in glioblastoma tumors express PD-L1 (50–90%), and PD-L1 positive cells dispersed in the specimen uniformly, while in the adjacent normal tissue in the tumor or normal sample, no expression of PD-L1 has been found (Fig. 2).

2. The functional correlation analysis of PD-L1 expression. Production of T cell cytokines and the inhibition of the expression of activation markers. To study the function and meaning of the PD-L1 expression derived from glioma cells, we cultured and purified the helper T cells with or without PD-L1 antibody using co-culture experiments. In addition to PD-L1, LN-229 glioma cells show a high expression of HLA-I composition formula and HLA-DR antigen. When the purified CD4⁺ T cells and LN-229 glioma cells were co-cultured, HLA-DR antigens and CD4⁺ T cell T cell receptors interacted, leading to the activation of T cells and the production of cytokines. Accordingly, HLA-I antigen of CD8⁺ T cells and glioma cells interact, leading to T cell activation. Cytokines (IFN-γ, IL-2 and

| Table 1 | Primers of 28srRNA and PD-L1. |
|---------|-------------------------------|
| Gene    | Sequence (5’-3’)              |
| 18s-for | CGGCTACCACATCCAAGGAA          |
| 18s-rev | GCTGGAATTACCGGCGCT            |
| PD-L1-for| TCAATGCCCCCATACAACAAA        |
| PD-L1-rev| TGCTTGCCAGATGACTTCG          |

Figure 1A Using real-time quantitative RT-PCR to detect the PD-L1 mRNA expression of 48 h cultured human glioma cells with or without IFN-γ (500 units/ml).

Figure 1B Using flow cytometry to analyze the PD-L1 protein expression of 48 h cultured human glioma cells with or without IFN-γ (500 units/ml).
IL-10) in the culture supernatants were detected by ELISA at predetermined release time points. Fresh not activated purified CD4\(^+\) and CD8\(^+\) T cells were co-cultured with glioma for 24 and 48 h. By adding anti-PD-L1 antibody, cytokines increased in co-culture supernatant (IFN-\(\gamma\): CD4\(^+\) T cells 310 ± 12.9\%, \(P = 0.017\); CD8\(^+\) T cells: 159 ± 8.0\%, \(P = 0.270\); IL-2: CD4\(^+\) 176 ± 15.3\%, \(P = 0.011\); CD8\(^+\) 146 ± 5.7\%, \(P = 0.338\); Table 2). PD-L1 took the most significant inhibition on the production of IFN-\(\gamma\) and IL-2 by CD4\(^+\) T cells. CD8\(^+\) T cells were also inhibited, but the production of cytokines did not reach statistically significant levels. The production of IL-10 varies between different donors. Generally, blockaded PD-L1 will increase the level of IL-10, but this effect is not significant (CD4\(^+\) T cells: 128 ± 5.3\%, \(P = 0.096\); CD8\(^+\) T cells: 119.2 ± 11.5\%, \(P = 0.201\)) (Table 2).

4. Discussion

A variety of immune evasion strategies prompt cancer progression. Glioma is a typical suppressed tumor with anti-tumor immune response, and the factors include TGF-\(\beta\), and IL-10 release, CD95L, CD70, or HLA-G (Wienandt et al., 2002) expression. Glioma immunotherapeutic strategies eliminate immunosuppressive mechanisms or strengthen the anti-tumor immune response (Paul and Kruse, 2001). In the present study we demonstrate that PD-L1, one of the B7 family of co-stimulatory molecules, composed the formulation of glioma lines (Fig. 1A). Immunohistochemical analysis showed that PD-L1 was expressed in brain tumor specimens, but did not express near the site of the tumor and normal human brain tissue (Fig. 2). In vitro co-culture experiments, we demonstrated that PD-L1 significantly impaired anti-tumor immune response to T cell cytokine production (see Table 2).

Under physiological and non-inflammatory conditions, PD-L1 is the main antigen presenting cell in the body, such as the expression (Carter et al., 2002) of monocytes and DCs. However, under inflammatory conditions, PD-L1 is no longer limited to antigen presenting cells, it is also expressed by other cells such as endothelial and muscle cells (Surhio et al., 2014).

Our study provides evidence of PD-L1 expression in the brain in vivo. All malignant gliomas express PD-L1, but it was not expressed near the site of the tumor and normal human brain tissue (Fig. 2). This is similar to the culture of glioma cell lines in vitro (Fig. 1A). It remains unclear how and when to express PD-L1’s by the glioma cells. In consistency with the previous results, RNA and protein levels of PD-L1 expression in glioma cells were significantly upregulated in inflammatory cytokines, such as IFN-\(\gamma\) stimulation, but there was no such effect in TNF-\(\alpha\) (Fig. 1A).

PD-L1, expressed by glioma, is an anti-immune response inhibitor. This has been proved by co-culture experiments in vitro. With the presence of anti-PD-L1 antibody, the production of IFN-\(\gamma\) and IL-2 by CD4\(^+\) and CD8\(^+\) T cells were significantly strengthened, and IL-10 is only slightly raised. Recent studies have shown that activated T cells stimulated by PD-L1 lead to apoptosis (Dong et al., 2002), and the proliferation of activated T cell and cytokine production will be inhibited by the combination of PD-1 and PD-L1 (Freeman et al., 2000; Carter et al., 2002). Recent studies also showed that the expression of PD-L1 in DCs exert an intense immunosuppressive effect on the activation of T cells. Thus, PD-L1 is an important molecule for induction and maintenance anergy of T cell under physiological conditions, which is conducive to the immune body from damage caused by excess immune response under normal physiological conditions, and to immunological tolerance to self-antigens (Brown et al., 2003; Seleno-Gebacker et al., 2003). In addition to the interaction between PD-1 and PD-L1, the inhibitory effect of PD-L1 can be mediated by other receptors (Naureen et al., 2014; Naureen et al., 2014).
Kiyani et al., 2014; Khaskheli et al., 2015). Our results support that PD-L1 plays a negative regulatory function on T cell activation on the whole. Glioma associated PD-L1 expression is a mechanism of immune suppression of tumor escape immune recognition and destruction in the CNS.

5. Conclusion

Glioma related inhibition of T cell in vivo by PD-L1 indicates that this co-stimulation molecule inhibits the T cell growth and cytokine production. Thus, PD-L1 glioma associated expression may affect the anti-immune response at the initial and secondary phase in vivo conditions. PD-L1 could inhibit T-cells at the start and DC functions to interfere with the primary anti-tumor immune response (Brown et al., 2003; Selenko-Gebauer et al., 2003; Curiel et al., 2003). Moreover, PD-L1 can protect glioma cells from directly attacking the antigen-specific cytotoxic T cells (Safi et al., 2015). The present results indicate that the effective blockage of the interaction between PD-1 and immune effector cells can be used as a potential therapeutic strategy for immunotherapy of glioma in vivo. It also means that the blocking of the PD-1/PD-L1 pathway has a potential value for immunotherapy of the glioma.

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