Expression of the B7-Related Molecule B7-H1 by Glioma Cells: A Potential Mechanism of Immune Paralysis

Sabine Winterle, Bettina Schreiner, Meike Mitsdoerffer, Dagmar Schneider, Lieping Chen, Richard Meyermann, Michael Weller, and Heinz Wiendl

Abstract

Human glioblastoma is a highly lethal tumor that is known for its immune inhibitory capabilities. B7-homologue 1 (B7-H1), a recently identified homologue of B7.1/2 (CD80/86), has been shown to exert costimulatory and immune regulatory functions. We investigated the expression and the functional activity of B7-H1 in human glioma cells in vitro and in vivo. Although lacking B7.1/2 (CD80/86), all 12 glioma cell lines constitutively expressed B7-H1 mRNA and protein. Exposure to IFN-γ strongly enhanced B7-H1 expression. Immunohistochemical analysis of malignant glioma specimens revealed strong B7-H1 expression in all 10 samples examined, whereas no B7-H1 expression could be detected on normal brain tissues. To elucidate the functional significance of glioma cell-related B7-H1 expression, we performed coculture experiments of glioma cells with allogeneic CD+ and CD8+ T cells. Glioma-related B7-H1 was identified as a strong inhibitor of CD8+ T-cell activation as assessed by increased cytokine production (IFN-γ, interleukin-2, and interleukin-10) and expression levels of the T-cell activation marker (CD69) in the presence of a neutralizing antibody against B7-H1 (mAb 5H1). B7-H1 expression may thus significantly influence the outcome of T-cell tumor cell interactions and represents a novel mechanism by which glioma cells evade immune recognition and destruction.

Introduction

Accumulating evidence indicates that specific T-cell immune responses can be raised against many tumors (1, 2). Nonetheless, tumor-specific immune responses are not sufficient to eradicate the tumors in the instance of clinical cancer (3, 4). Antigen-induced activation and proliferation of T cells are regulated by both positive and negative costimulatory receptors of the immunoglobulin (Ig) superfamily (5, 6). Abrogation of positive costimulatory activity, mediated by, e.g., B7.1/2/CD28 or ICOS ligand/ICOS, using monoclonal antibodies and soluble receptors that neutralize costimulatory molecules or targeted disruption of gene expression, results in compromised cellular and humoral immune responses. This can be beneficial in the context of autoimmune disease and transplant rejection (7, 8). The lack of augmentative costimulatory activation can be detrimental, however, if immune responses against cancer are compromised (9). Human glioblastoma is a highly lethal tumor that is paradigmatic for its ability to suppress effective antitumor immune responses (10). The identification of negative immune regulatory signals on tumor cells, however, has given rise to the hypothesis and hope that their manipulation may lead to enhanced tumor-specific T-cell immunity in vivo.

B7-H1, a recently identified homologue of B7.1/2 (CD80/86), has been shown to exert costimulatory and immune regulatory functions (5, 6, 11, 12). The costimulation of T cells with B7-H1-Fc fusion protein induces T-cell proliferation and the preferential secretion of IL-10 and IFN-γ (13). Other laboratories, however, showed that B7-H1-Fc inhibited cytokine synthesis because of cell cycle arrest (14, 15). PD-1, a receptor for B7-H1, contains an immune receptor tyrosine-based inhibitory motif, and coligation of PD-1 and the T-cell receptor leads to rapid phosphorylation of Src homology region 2-containing protein tyrosine phosphatase-2, a phosphatase suggested to attenuate T-cell receptor signaling (16).

We here report that glioma cells express high levels of B7-H1 in vitro and in vivo and that B7-H1 expressed on glioma cells reduces their immunogenicity in vitro. B7-H1 is thus a novel mediator that may contribute to the immune inhibitory characteristics of human glioma.

Materials and Methods

Antibodies and Reagents. The following primary antibodies were used: antihuman B7-H1 (clone 5H1; Ref. 17); anti-HLA (human leukocyte antigen)-A2 (BB7.2; American Type Culture Collection, Manassas, VA); anti-CD8+ FITC (B9.11; Immunotech, Marseille, France); anti-HLA class I (W6/32); anti-HLA-DR (L243); anti-CD69-FITC (CH/4; CALTAG Laboratories, Hamburg, Germany); anti-CD14 (B-A8); anti-CD20 (B9E9); anti-CD56 (anti-NCAM, MY31); anti-CD4+ FITC (RPA-T4); anti-CD80 (BB1); anti-CD86 (B-T7 all from BD Pharmingen, Heidelberg, Germany); antihuman ICOS; and antihuman ICOS-PE (F44, generously provided by Richard A. Krowczek, Robert-Koch-Institut, Berlin, Germany). Secondary antibodies and isotype controls were: goat antimouse F(ab')2-dil-chlorotriazinyl-fluorescein; goat antimouse-PE IgG (H+L) F(ab')2 fragment (Dianova, Hamburg, Germany); mouse IgG1-FITC; mouse IgG1-PE (MOPC-21; Sigma, St. Louis, MO); mouse IgG (Linearis, Wertheim, Germany); IgG1x (MOPC-31C and 107.3); and IgG2a (G155-178; BD Pharmingen). IFN-γ and TNF-α were from PeproTech EC Ltd. (London, United Kingdom). Normal goat serum was from Dianova (Hamburg, Germany), human IgG (Alphaglobin) was obtained from Grifols (Langen, Germany), and CD3/CD28 beads were from Dynal Biotech (Hamburg, Germany).

Cell Culture. The human malignant glioma cell lines LN-18, U138MG, U87MG, LN-428, D247MG, T98G, LN-319, LN-229, A172, U251MG, U373MG, and LN-308, kindly provided by Dr. Nicolas de Tribolet (Lausanne, Switzerland), were cultured as described (18). TEB71 cells were obtained from American Type Culture Collection.

Immunohistochemistry. Tumor specimens were surgically removed from 9 patients with glioblastoma (WHO grade IV) and 1 patient with a mixed glioma (WHO grade III, female, n = 5, age range, 28–89 years, median, 68 years; male n = 5, age range, 60–71 years, median, 64 years). One biopsy of normal brain tissue and the normal brain tissue in proximity to the neoplastic cells were used as control tissues. Normal human thymuses were obtained following Institutional Review Board guidelines from 2-day- to 14-year-old children undergoing corrective cardiac surgery. Immunohistochemistry was performed as described previously (18). In brief, frozen sections (20 µm) were cut, fixed in acetone, and immunostained with anti-B7-H1 antibody (5H1) or
an isotype control antibody. The reaction product was visualized with the streptavidin-biotin reagent (Dako, Hamburg, Germany) using 3,3'-diaminobenzidine (Serva, Heidelberg, Germany) as a substrate. Biopsy specimens stained with immunohistochemistry were assessed independently by two experimenters. The percentage of positively stained glioma cells was quantified from <25, 25–50, 50–75, and >75%.

PBMCs, Purified Lymphocyte Populations, and DCs. PBMCs were isolated from the peripheral blood of normal healthy volunteers (18). CD4+ and CD8+ T cells (>95%) were purified using microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Coculture experiments were carried out as described previously (18). Cytokines released into the supernatants (human IFN-γ, IL-2, and IL-10) were measured by ELISA (BD Pharmingen). The expression of T-cell activation markers was assessed by flow cytometry. Monocytes were obtained from Ficoll-separated PBMCs of healthy volunteers. B cells were removed using CD22+ magnetic beads (Miltenyi Biotec), and monocytes were obtained after 1 h adherence step in RPMI 1640 containing 10% FCS at 37°C. Monocytes, >90% pure as assessed by flow cytometry, were cultured in RPMI 1640 containing 10% FCS supplemented with granulocyte-macrophage colony-stimulating factor (100 ng/ml, Leukomax; Sandoz, Basel, Switzerland) and IL-4 (40 ng/ml; PeproTech, Inc., Offenbach, Germany). After 6 days the cells exhibited an immature DC phenotype (CD14− and CD14+ and HLA-DRlow and CD86low and D8low/− and CD83−). Maturation was induced by incubation of the immature DCs with lipopolysaccharide (5 μg/ml, S. typhii; Sigma L-7261) or TNF-α (200 units/ml; PeproTech EC Ltd). High levels of surface HLA-DR and costimulatory molecules (CD86 and CD80) identified mature DCs.

Flow Cytometry. Surface expression of immune molecules was quantified by flow cytometry on a fluorescence activated cell sorter (FACS caliber cytometer; Becton Dickinson, Heidelberg, Germany) using CellQuest software. A total of 10,000 events/antigen was evaluated. Histograms were analyzed by calculating the SFI (geometric mean of the specific antibody fluorescence divided by the geometric mean of the isotype control antibody fluorescence).

Coculture Experiments. Coculture experiments were carried out as described previously (19). Glioma cells were plated in 48-well plates (Costar, Baden, Germany) at a density of 5 × 10⁴ cells/well and cultured in the absence or presence of IFN-γ (500 units/ml, 48 h) by QRT-PCR. All glioma cell lines expressed low levels of B7-H1 mRNA constitutively. In the presence of IFN-γ, B7-H1 expression was increased by 1.7-fold (LN-428) to 75-fold (LN-319; mean 21.64 ± 21.68 SD; Fig. 1A). In contrast to IFN-γ, TNF-α had no effect on B7-H1 mRNA expression (data not shown). Flow cytometry revealed constitutive B7-H1 cell surface expression in all 12 glioma cell lines examined. After stimulation with IFN-γ, B7-H1 expression was enhanced 1.1-fold (LN-428) to 9.3-fold (D247MG) as assessed by SFI ratio (Fig. 1B). The expression of HLA class I antigens, HLA-DR antigens, B7.I (CD80) and B7.2 (CD86), and their inducibility by IFN-γ were assessed in parallel. Although constitutively expressing HLA class I antigens, inducibly or constitutively expressing HLA-DR antigens, glioma cells lack B7.1/2 (CD80/86) mRNA and protein [Table 1, Fig. 2; (Refs. 18, 20)]. In contrast to the tested glioma cells, TE671, a rhabdomyosarcoma cell line, showed no constitutive expression of B7-H1 mRNA or protein (Fig. 1A).

B7-H1 Expression by Glioma Cells in Vivo. Using immunohistochemistry, B7-H1 protein expression was detected in all 9 glioblastoma specimens (WHO IV) and 1 mixed glioma (WHO III) specimen. More than 50% of the tumor cells expressed B7-H1 in the glioblastoma specimens (range, 50–90%). B7-H1-positive cells were evenly scattered throughout the specimens. In contrast, no B7-H1 was found in normal brain tissue adjacent to tumor tissue or in normal brain biopsy specimen. Human thymus was used as a positive control (Ref. 17; Fig. 3).

Functional Relevance of B7-H1 Expression: Inhibition of T-Cell Cytokine Production and Expression of Activation Markers. To investigate the functional significance of endogenous B7-H1 expression on glioma cells, we performed coculture experiments with purified T-helper cell subsets in the absence and presence of a blocking antibody against B7-H1. In addition to B7-H1, LN-229 glioma cells exhibit high constitutive expression levels of HLA class I and HLA-DR antigens but do not express CD80 and CD86 (Table 1, Figs. 1, 2, and 4A). Upon coculture of purified CD4+ T cells with LN-229 glioma cells, HLA-DR antigens interact with the T-cell receptor on CD4+ T cells, leading to T-cell activation and cytokine production. Correspondingly, alloreactive CD8+ T cells interact with HLA class I antigens on glioma cells, leading to T-cell activation. The release of cytokines (IFN-γ, IL-2, and IL-10) into the culture supernatant was assessed by ELISA at times indicated. Freshly purified, alloreactive unprimed CD4+ and CD8+ T-cell subsets were cocultured with the glioma cells for 24 or 48 h. In the presence of anti-B7-H1 antibody, cytokine production was enhanced in cocultures of glioma cells and CD4+ or CD8+ T cells (relative change in cytokine production for IFN-γ: CD4+ T cells 310 ± 53.9%, P = 0.017; CD8+ T cells: 159 ± 46.1%, P = 0.270; IL-2: CD4+ 176 ± 16.8%, P = 0.011; CD8+ 150 ± 46.4%, P = 0.338; Fig. 4A). The inhibitory effects of B7-H1 on IFN-γ and IL-2 production were most prominent for CD4+

Results

B7-H1 Expression in Human Glioma Cell Lines. Twelve human glioma cell lines were tested for B7-H1 mRNA expression in the absence or presence of IFN-γ (500 units/ml, 48 h) by QRT-PCR. All glioma cell lines expressed low levels of B7-H1 mRNA constitutively. In the presence of IFN-γ, B7-H1 transcription was increased from 53.9% to 75% (mean 21.64 ± 21.68 SD; Fig. 1A). In contrast to IFN-γ, TNF-α had no effect on B7-H1 mRNA expression (data not shown). Flow cytometry revealed constitutive B7-H1 cell surface expression in all 12 glioma cell lines examined. After stimulation with IFN-γ, B7-H1 expression was enhanced 1.1-fold (LN-428) to 9.3-fold (D247MG) as assessed by SFI ratio (Fig. 1B). The expression of HLA class I antigens, HLA-DR antigens, B7.1 (CD80) and B7.2 (CD86), and their inducibility by IFN-γ were assessed in parallel. Although constitutively expressing HLA class I antigens, inducibly or constitutively expressing HLA-DR antigens, glioma cells lack B7.1/2 (CD80/86) mRNA and protein [Table 1, Fig. 2; (Refs. 18, 20)]. In contrast to the tested glioma cells, TE671, a rhabdomyosarcoma cell line, showed no constitutive expression of B7-H1 mRNA or protein (Fig. 1A).

Functional Analysis of B7-H1 Expression. Oligonucleotides used in this study are listed below: 18S, 18s-for 5′-GGCTTACACATCAGAAAGA-3′ and 18s-rev 5′-GCCGACTTCGATTCCGGCT-3′; HLA-DR, HLA-DR-a-for 5′-GAAATTGGGACATTGCCGTC-3′ and HLA-DR-a-rev 5′-GGAGGTGTCATTTGGTACCG-3′; and B7-H1, B7-H1-for 5′-TCAATGGCCCATACCAAAAA-3′ and B7-H1-rev 5′-TGTTGGTCCAGATGCTTCG-3′. Statistical Analysis. Statistical analysis was performed with one sample t test (*, P < 0.05; **, P < 0.01).

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T cells. CD8+ T cells were also inhibited, but the changes in cytokine production did not reach the level of statistical significance. The effects on IL-10 production differed between different donors. In general, B7-H1 blockade increased IL-10 levels in the cocultures, but this effect was not significant (CD4+ T cells: 154.8 ± 25.3%, P = 0.096; CD8+ T cells: 119.2 ± 12.6%, P = 0.201). Anti-HLA-DR mAb L243 and anti-HLA-A, HLA-B, and HLA-C mAb W6/32 were used as positive controls to suppress HLA class I- and HLA-DR-restricted activation and cytokine production by CD4+ and CD8+ T cells (Fig. 4A).

The B7-H1-mediated inhibition of cytokine production was paralleled by corresponding changes in the expression of the T-cell activation marker CD69. CD4+ and CD8+ T cells were cocultured with LN-229 cells in the absence or presence of anti-B7-H1 mAb and subsequently stained for T-cell activation markers CD69 and ICOS. Inhibitory effects of endogenous B7-H1 on T-cell activation were documented by increased levels of CD69 in the presence of anti-B7-H1 mAb. In contrast, there was no effect on the expression of ICOS (Fig. 4B). Similar experiments were performed with another glioma cell line, T98G. In addition to B7-H1, T98G glioma cells exhibit high constitutive expression levels of HLA class I but do not express CD80, CD86, or HLA-DR antigens (Fig. 1, Table 1). Before the coculture experiments, glioma cells were pretreated with IFN-γ (500 units/ml, 24 h) to up-regulate HLA-DR antigen and B7-H1 expression. Similar data on the inhibitory effects of B7-H1 on CD4+ and CD8+ T-cell cytokine production and activation were achieved (data not shown).

**Table 1** Expression of HLA class I and HLA-DR antigens in glioma cell lines in the absence or presence of IFN-γ

<table>
<thead>
<tr>
<th>Glioma cell line</th>
<th>HLA class I</th>
<th>IFN-γ</th>
<th>IFN-γ</th>
<th>HLA-DR mRNA</th>
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<tr>
<td></td>
<td>- IFN-γ</td>
<td>IFN-γ</td>
<td></td>
<td>Absorbance</td>
</tr>
<tr>
<td>A172</td>
<td>21.9</td>
<td>17.2</td>
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<td>6.3</td>
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<td>T98G</td>
<td>191.1</td>
<td>202.2</td>
<td>1.0</td>
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<td>134.3</td>
<td>271.9</td>
<td>193.5</td>
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<tr>
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<td>148.0</td>
<td>1.0</td>
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<td>52.6</td>
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<td>5.3</td>
</tr>
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<td>43.9</td>
<td>1.0</td>
<td>5.5</td>
</tr>
<tr>
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<td>16.4</td>
<td>33.6</td>
<td>1.0</td>
<td>5.8</td>
</tr>
<tr>
<td>LN-308</td>
<td>5.2</td>
<td>13.5</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
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<td>105.0</td>
<td>240.0</td>
<td>1.0</td>
<td>4.3</td>
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<tr>
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<td>43.9</td>
<td>1.0</td>
<td>5.3</td>
</tr>
<tr>
<td>U87MG</td>
<td>8.0</td>
<td>15.8</td>
<td>1.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

*Protein expression of HLA class I and HLA-DR antigens was quantified by flow cytometry using anti-HLA class I mAb W6/32 and anti-HLA-DR mAb L243 and expressed as SFI [modified from (18)]. mRNA expression of HLA-DR-α was quantified by relative QRT-PCR. TNF-α-matured DCs were used as a positive control and set to 100% (data not shown). Data are expressed as a percentages relative to this positive control.
Our study provides the first evidence for the expression of B7-H1 within the brain in vivo. All malignant glioma specimens expressed B7-H1, whereas normal CNS tissue adjacent to the neoplastic cells and one cortical biopsy without pathological alterations did not exhibit B7-H1 immunoreactivity (Fig. 3). This observation was paralleled by the demonstration of strong constitutive B7-H1 expression in all tested cultured glioma cell lines (Fig. 1). It remains elusive, at present, how and when glioma cells acquire B7-H1 expression. Consistent with previous studies (e.g., Ref. 13), RNA and protein levels of B7-H1 were additionally up-regulated in the presence of inflammatory cytokines such as IFN-γ, whereas TNF-α had no such effect (Fig. 1). These data corroborate and extend two recent studies showing B7-H1 expression on melanoma cells and carcinoma cells of lung, ovary, and colon, as well as in some tumor cell lines of non-CNS origin (17, 31). More detailed investigations are warranted to elucidate the involvement of B7-H1 and its receptor interactions in other inflammatory and noninflammatory CNS pathologies.

Glioma-related B7-H1 expression was identified as a strong inhibitor of antitumor immune responses as assessed under alloreactive coculture conditions in vitro. In the presence of a neutralizing antibody to B7-H1 (mAb SH1), the levels of IFN-γ and IL-2 produced by CD4+ and CD8+ T cells were markedly enhanced, an observation that paralleled increased expression of the T-cell activation marker CD69 (Fig. 4). Under certain experimental conditions, B7-H1 in the form of an immunoglobulin (Ig) fusion protein (B7-H1-Ig) or cell-associated B7-H1 together with a mAb against CD3 can exert costimulatory functions on T cells in vitro (13, 32). However, recent studies indicate that prolonged B7-H1 stimulation of activated T cells leads to increased apoptosis (17) and that ligation of PD-1 by B7-H1 inhibits proliferation and cytokine production by activated T cells (14, 15). It has also recently been shown that B7-H1 expressed on DCs exerts strong immune inhibitory effects on autologous T-cell activation. B7-H1 has therefore been proposed as an important principle involved in the induction and maintenance of T-cell anergy under physiological conditions (31, 33). Furthermore, in addition to B7-H1-PD-1 interactions, inhibitory effects of B7-H1 are mediated via yet unidentified non-PD-1 receptors (17, 34). Our study is in line with the concept of an overall negative regulatory function of B7-H1 for T-cell activation and proposes glioma-related B7-H1 expression as a potential mechanism of immune inhibition in the CNS. B7-H1 expressed on...
A glioma cells probably inhibits T-cell activation by engagement of a non-CD1 receptor because mAb 5H1, the neutralizing antibody used in our blocking experiments (Fig. 4), does not interfere with the binding of B7-H1 to PD-1 receptor (34).

Tumor-associated B7-H1 expression was recently shown to promote T-cell apoptosis and proposed as a potential mechanism of immune evasion by certain non-CNS tumors (17, 35). B7-H1-mediated induction of T-cell apoptosis may be executed by multiple mechanisms such as triggering IL-10 secretion or involvement of CD95L (17). However, under our experimental conditions, we did not detect a direct proapoptotic effect of glioma-related B7-H1 on CD4+ or CD8+ cells (data not shown). The use of different experimental systems investigating the significance of tumor-associated B7-H1 expression may explain these differences. Tumor antigen-specific T-cell clones were used by Dong et al. (17), whereas our experimental approach aimed at demonstrating the relevance of B7-H1 for glioma immune cell interactions under primary alloreactive coculture conditions. T-cell clones are known for their high susceptibility to activation-induced cell death, a mechanism that is of minor importance in our experimental system. Along with this, the ability to induce apoptosis in freshly isolated lymphocytes depends on the level of preactivation. Unstimulated responder cells were cocultured with B7-H1-bearing glioma cells here, thus reducing their susceptibility to apoptosis. Of note, the influence of B7-H1 on IL-2 production differed between CD4+ and CD8+ T cells (Fig. 3). This can potentially be explained by the different requirements on costimulation between CD4+ and CD8+ T cells. Although there is consensus on the necessity of a costimulatory signal for CD4+ T-helper functions such as T-cell proliferation promoted by IL-2, the influence of costimulation on CD8+ cytotoxic T cells for the exertion of effector functions is less clear.

Our in vitro data concerning T-cell inhibition by glioma-related B7-H1 indicate that this costimulatory molecule inhibits T-cell growth and cytokine production. Therefore, glioma-related B7-H1 expression is likely to influence both primary and secondary phases of antitumor immune responses under in vivo conditions. B7-H1 could interfere with mounting primary antitumor immune responses via inhibition of T-cell priming and DC function (14, 36). Furthermore, B7-H1 could protect glioma cells from direct immune attack by antigen-specific cytotoxic T cells at the effector level (17, 35). In any event, the present results strongly suggest that effective blockade of B7-H1 interactions with immune effector cells in vivo should provide a promising strategy of immunotherapy for selected tumors expressing B7-H1.

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