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Gamma interferon mediated superinduction of B7-H1 in PTEN-deficient glioblastoma: A paradoxical mechanism of immune evasion

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Abstract

B7-H1 is a recently discovered immunoresistance protein that is regulated post-transcriptionally after PTEN loss in malignant glioma, a deadly form of brain tumor. Here, the impact of gamma interferon mediated activation of B7-H1 was investigated in glioblastoma patients with PTEN loss. Lymphocytes and T-cells were selected for apoptosis assays after 1:1 co-culture with autologous glioma cells. Gamma interferon treatment of PTEN deficient tumors resulted in superinduction of B7-H1 protein that correlated with increased T-cell apoptosis, an effect dependent upon activation of the PI3Kinase pathway. The combination of PTEN loss and gamma interferon exposure in glioblastoma patients results in an exceptionally immunoresistant phenotype that may negate adaptive immunity through induction of T-cell apoptosis.

Keywords

B7-H1; PD-L1; Interferon gamma; Glioma; Immunoresistance; Immune evasion

Introduction

A key challenge in the development of immunotherapeutic modalities for malignant glioma has been overcoming local immunoresistance and systemic immunosuppression [1]. For example, B7 homolog 1 (B7-H1), also known as programmed death ligand-1 (PD-L1), induces apoptosis of activated T-cells and hinders tumor specific killing by cytotoxic T-cells when expressed on the cell surface of glioma [2]. While normal human cells contain B7-H1 transcript, they usually express little or no B7-H1 protein [2–4]. In malignant glioma, B7-H1 protein is expressed at high levels [5–10], with a correlation between level of B7-H1 protein expression and tumor grade [10]. Deficiency of the tumor suppressor gene, phosphatase and tensin homolog (PTEN) function results in increased expression of the B7-H1 protein through a well defined post-transcriptional mechanism that is dependent on the PI(3) kinase (PI3K) pathway and S6 kinase activation [11].

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γ -Interferon (IFN- γ) is a powerful immunomodulatory cytokine secreted by activated T-cells. IFN- γ increases immunogenicity of target cells, including glioma cells, by upregulation of MHC Class I and Class II expression on gliomas [12, 13]. Paradoxically, a potential pathway to immunoresistance conferred by IFN- γ in context of human glioma has been demonstrated by Wilmotte and colleagues, who noted that the expression of B7-H1 protein is partly IFN- γ dependent [10]. In this study we hypothesized that an increase in B7-H1 transcript after IFN- γ treatment would result in superinduction of B7-H1 protein in patients with PTEN loss, mediated in part by post-transcriptional regulatory mechanisms, conferring an extremely immunoresistant phenotype.

Materials and Methods

Immunohistochemistry

Immunohistochemistry was performed at the UCSF Department of Pathology. Hematoxylin and Eosin and PTEN stains were performed on Paraffin blocks of tumor samples processed on BenchMark XT (Ventana Medical Systems, Tucson, AZ). Rabbit anti-human PTEN antibody (Cell Signaling, Danvers, MA) was used at 1:100 dilution. The average percentage of tumors cells with positivity to PTEN stain of three high power fields was measured to categorize the tumor into the four widely accepted PTEN score categories: 0–25%, 25–50%, 50–75%, and 75–100% [14].

Normal Human Astrocyte (NHAs) Cell Line Preparation

Retroviral transfection of NHAs (Clonetics) produced cells with genetic alterations functionally equivalent to those in human malignant gliomas. Immortalized hTERT/E6/E7 cells were produced by introduction of pWZL-blast-hTERT and pLXSP-puro-E6/E7. Ras+ cells were generated by independent retroviral introduction of pLXSN-neo-H-RasV12. Akt+ cells and Ras+/Akt+ cells were created by independent retroviral infections of Ras+ cells with pWZL-hygro-myrAktdelta4-129.

Glioma Cell Cultures

Well characterized glioma cell lines, U87 and SF767 were obtained from the University of California, San Francisco (UCSF) Brain Tumor Research Center, and cultured in Dulbecco's Modified Eagle Medium (DME H-21) with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin.

Primary human glioblastoma cell lines were derived from tumor samples that were resected from patients treated at UCSF Medical Center, with the approval of the UCSF Medical Center Institutional Review Board and Committee on Human Research. Glioma cells were isolated by mechanical and enzymatic dissociation, followed by density separation by Percoll (Sigma-Aldrich) gradient. Twenty six different GBM lines were established and cultured in RPMI-1640 25 M Hepes and 2.0 g/L NaHCO₃, with 25% FBS, 1% penicillin-streptomycin, 1 mM sodium pyruvate, 10 mM non-essential amino acids.

Isolation of Peripheral Blood Lymphocytes (PBLs) and T-cells

PBLs were isolated from whole blood of patients collected at time of surgery by density gradient using Ficoll-Paque (GE HealthCare). From six patients, T-cells were isolated from the population of PBLs using the CD3 Human T-cell Enrichment Kit (Stem Cell Technologies). T-cells were cultured in complete T-cell medium. RPMI-1640 25 M Hepes and 2.0 g/L NaHCO₃, with 10% FBS, 1% penicillin-streptomycin, 1 mM sodium pyruvate, 10 mM non-essential amino acids and expanded by stimulation with anti-CD3 (2 μ g/mL) and anti-CD28 (5 μ g/mL) antibodies, (eBioscience).

Treatment with Gamma Interferon and Inhibitors of the PI3K Pathway

NHAs and tumor cells (1×10^5 cells/mL) were treated with recombinant human IFN- γ (R&D Systems) at 100 units/ mL for 18 hours, and/or with one of the following 4 inhibitors of the PI3K pathway for 48 hours: AKT Inhibitor III (AKT III) at 50 μ M (Calbiochem), LY 294002 (LY294) at 100 μ M (Calbiochem), Rapamycin (Rapa) at 100 μ M (Calbiochem), and Honokiol (HNK) at 20 μ M (Dr. Jack Arbiser, Emory University) [15]. Different steps of the PI3K pathway were targeted: PI3K by LY 294002, Akt by AKT Inhibitor III and Honokiol, and mTOR by Rapamycin.

Cytotoxic Assays

Tumor cells treated with or without IFN- γ and/or PI3K pathway inhibitors were plated at 1×10^5 cells/mL and allowed to adhere; next, stimulated autologous PBLs or T-cells were added to the same patients' tumor cells. Cells were co-cultured for 18 hours, and prepared for flow cytometric analysis. As positive control for apoptosis, cells treated with 1 μ M staurosporine, a non specific kinase inhibitor, (Sigma Aldrich) were used. On flow cytometric analysis cell viability was monitored by gating on cell size and granularity and counting of cells that were CD45 positive, but Annexin V negative.

Quantitative PCR

mRNA of B7-H1 was obtained from tumor cells using RNeasy Mini Kit (Qiagen) and cDNA was generated using Superscript III Kit (Invitrogen). Transcript levels were detected using SYBR Green (Applied Biosystems) and the CFX96 Real-Time System (Bio-Rad Laboratories). Values of transcript levels were standardized using hypoxanthine phosphoribosyltransferase (HPRT), which has been shown not to be transcriptionally regulated by IFN- γ [16]. Sequences of primers used were: B7-H1 (5'-GCTGTT-GAAGGA-CCAGCT-CT/TGCTTG-TCCAGA-TGACTT-CG-3') and HPRT (5'-GACCAG-TCAACA-GGGGAC-AT/CCTGAC-CAAGGA-AAGCAA-AG-3').

Flow Cytometry

Tumor samples were stained with FITC conjugated HLA-ABC (BD Biosciences) and PE B7-H1 (eBioscience). Samples from co-culture were stained with APC conjugated CD45 (eBioscience) and PE Annexin V (BD Biosciences). Flow cytometry data was gathered on FACSCaliber Flow Cytometer using CellQuest Pro software (BD Biosciences), and analyzed with FlowJo software (Treestar).

Statistical Analysis

For two sample comparisons, the 2-tailed student t-test was used with statistical significance at the p-value of < 0.05 . For multiple comparisons, analysis of variance (ANOVA) was used. Significance levels for multiple comparison tests were adjusted using the Bonferroni correction. Data for figures were collected from independent experiments performed in triplicate. Representative experimental data are shown for B7-H1 qPCR, B7-H1 flow cytometry, and T-cell apoptosis experiments. Error bars represent standard deviation, unless otherwise stated.

Results

Previous reports have established that immortalized NHAs do not differ in their expression levels of B7-H1 protein from their normal counterparts [11]. Here, NHAs transfected with E6/E7 did not show a significant increase in level of B7-H1 protein expression when treated with IFN- γ , while both Ras+ NHAs and Akt+ NHAs showed a significant increase in B7-H1

protein expression levels with IFN- γ treatment (Fig. 1). Exposure to IFN- γ resulted in a dramatic increase in B7-H1 protein levels in Ras+/Akt+ NHAs (Fig. 1).

In vivo, glioblastoma multiforme (GBM) cells show a range of functional PTEN expression (Fig. 2B,D), and loss of PTEN is correlated with activation of the PI3K pathway and increased B7-H1 expression [11]. Based on immunohistochemical findings of PTEN levels, established glioma cell lines were categorized as PTEN deficient (PTEN-), if positive staining was found in 0–25% of cells (2/6 cell lines) or in 25–50% of cells (1/6). Tumors were considered PTEN intact (PTEN+), if positive staining was found in 50–75% of cells (1/6) or in 75–100% of cells (2/6).

Transcript levels of B7-H1 were not significantly different in PTEN- tumors compared to PTEN+ tumors at baseline (Fig.3A). In contrast, expression levels of B7-H1 protein were significantly higher in PTEN- tumors compared to PTEN+ tumors at baseline (Figure 3B). Treatment with IFN- γ resulted in increased B7-H1 transcript and protein expression in both PTEN- and PTEN+ cell lines (Fig. 3A,B). In addition, IFN- γ induced a significantly larger degree of increase in levels of B7-H1 protein and transcript in PTEN- tumor cells than in PTEN+ tumors (Fig. 3A,B).

To investigate the functional consequences of the observed superinduction of B7-H1 in PTEN- tumors by IFN- γ , the degree of apoptosis of autologous lymphocytes and T-cells were analyzed in co-culture experiments. Degree of apoptosis was measured by positivity of Annexin V staining using flow cytometry. Proportions of viable cells were determined by those with normal size and granularity, negativity of Annexin V stain and positivity for CD3 +/- CD45. As illustrated in Fig. 3C, co-culture of autologous lymphocytes with PTEN- tumor cells resulted in a significantly higher degree of lymphocyte apoptosis than co-culture of lymphocytes with autologous PTEN+ tumor cells (mean % apoptosis: 45% and 12%, respectively; $p < 0.001$). Co-culture with autologous PTEN- tumor cells treated with IFN- γ demonstrated a significantly increased degree of lymphocyte apoptosis (mean % apoptosis from 45% to 72%; $p < 0.001$), along with decreased subsets of viable lymphocytes. For samples from patients with PTEN+ tumor, co-culture experiments after treatment of tumors with IFN- γ did not demonstrate a significantly higher degree of lymphocyte apoptosis.

After co-culture with autologous tumor cells, T-cells of patients with PTEN- tumors had equivalent levels of tumor-induced apoptosis as T-cells derived from patients with PTEN+ tumors (Fig. 3D). Compared to co-culture with tumors without treatment of IFN- γ , T-cells after co-culture with autologous PTEN- tumor cells treated with IFN- γ resulted in a significantly increased level of apoptosis (23% to 64%; $p < 0.001$). After IFN- γ treatment there was also a decrease in proportion of viable T-cells (data not shown). This increase in apoptosis was not observed for co-culture experiments utilizing samples from PTEN+ glioma patients.

To determine if the effects of IFN- γ seen above on levels of B7-H1 protein and T-cell apoptosis are mediated by activation of the PI3K pathway, B7-H1 protein measurements using flow cytometry and T-cell killing assays were repeated with tumors after treatment with inhibitors of the PI3K pathway, AKT III, LY294, Rapa, and HNK. For PTEN- samples, inhibition of the PI3K pathway decreased the B7-H1 levels of IFN- γ treated tumor cells to levels prior to treatment with IFN- γ (Fig. 4A). The percentage of T-cells undergoing apoptosis after co-culture with autologous PTEN- glioma cells treated with IFN- γ was greatly reduced by treatment of any of the four PI3K inhibitors prior to co-culture (Fig. 4C).

Discussion

In the current report, we studied the induction of B7-H1 protein expression by IFN- γ in the context of PTEN loss and PI3K pathway activation. In normal astrocytes with activated downstream targets of the PI3K pathway and tumor cell lines deficient of PTEN, treatment with IFN- γ resulted in a superinduction of B7-H1 protein. IFN- γ also induces a strong immunoresistant phenotype in glioma cells, as seen in lymphocyte and T-cell apoptosis assays and autologous co-culture experiments. In PTEN deficient gliomas, which have a greater degree of immune evasion in part due to B7-H1, this immunoresistant phenotype is more pronounced through the IFN- γ mediated B7-H1 superinduction and the associated increase in T-cell apoptosis. Considering that the two observed effects are both abrogated by PI3K pathway inhibition, our observations identify the activated oncogenic PI3K pathway as a potential therapeutic target for adjuvants to immunotherapies.

Conclusion

Here we demonstrate that the immunoresistant protein B7-H1 is dramatically upregulated after IFN- γ treatment of PTEN deficient glioma cells, with associated increases in lymphocyte and T-cell apoptosis in co-culture. Our report suggests that this IFN- γ mediated superinduction of B7-H1 protein contributes to the immune-evasive phenotype of human glioma, identifying a subset of glioma patients who are particularly resistant to T-cell based immunotherapeutic modalities.

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Figure 1.

B7-H1 protein levels are elevated by interferon- γ on astrocytes with Akt and Ras activation. Standardized fluorescence intensity of B7-H1 protein expression on the surface of human astrocytes immortalized by hTERT, E6, and E7 (E6/E7), immortalized and modified by Ras (Ras), Akt (Akt+), or both (Ras+ Akt+). (NS, nonsignificant; * $p < 0.05$)

Figure 2.

Variation in PTEN expression in human malignant glioma. (A) Patient A, Hematoxylin and Eosin, 100 $\times$, showing glioblastoma. (B) Patient A, PTEN stain, 100 $\times$, shows little to no positivity to stain using Rabbit anti-human PTEN antibody. Positive staining in vessels serves as positive control. 0–25% of cells show positivity to PTEN. (C) Patient B, Hematoxylin and Eosin 100 $\times$, showing glioblastoma. (D) Patient B, PTEN stain 100 $\times$, shows strong diffuse positivity to stain against PTEN. 75–100% of cells show expression of PTEN.

Figure 3.

PTEN loss leads to superinduction of B7-H1 in response to interferon- γ through transcriptional and translation regulation. (A) Representative B7-H1 transcript levels relative to HPRT in PTEN deficient (PTEN -) and PTEN wildtype (PTEN +) primary glioma lines before and after treatment with interferon- γ (+ IFN) (* $P < 0.001$) (NS, non-significant) (B) Standardized fluorescence intensity of B7-H1 protein expression of PTEN - and PTEN + primary glioma lines before and after treatment with interferon- γ (+ IFN). (* $P < 0.01$) (C and D) Loss of PTEN function and interferon- γ treatment of glioma cells increase apoptosis of autologous lymphocytes and T-cells in co-culture. (C) Co-cultures of lymphocytes with autologous tumors cells were analyzed for lymphocyte apoptosis by positivity to Annexin V staining. Staurosporin treated lymphocytes were used as positive control (not shown). Bars represent mean of 22 experiments, each from a unique patient. Error bars represent 95% confidence interval. (* $P < 0.001$) (D) Co-cultures of T-cells with autologous tumors cells were analyzed for T-cell apoptosis by measuring % of total CD45+ population that showed

positivity to Annexin V staining. Bars represent mean of triplicate samples from a representative experiment. Error bars represent $\pm$ SD. (NS, non-significant; * $P < 0.01$)

Figure 4.

Inhibition of PI3K pathway inhibits induction of B7-H1 and enhanced T-cell killing by interferon- γ . (A) Representative standardized fluorescence intensity of B7-H1 protein levels in PTEN deficient glioma cells treated with PI3K pathway inhibitors. PTEN deficient glioma cell line U87 and PTEN intact glioma cell line SF767 were used as positive and negative controls, respectively. (* $P < 0.0125$, relative to tumor + IFN) (B) Representative flow cytometry of co-culture stained with CD45 and Annexin V. Represented co-cultures include T-cells with autologous glioma cells (T cell, Tumor), autologous glioma cells treated with interferon- γ (T cell, tumor, IFN), or autologous glioma cells previously treated with interferon- γ and rapamycin (T cell, tumor, IFN, Rapamycin. All samples are derived from

PTEN deficient glioma patients. (C) T-cell apoptosis in presence of PTEN deficient gliomas treated with interferon- γ and PI3K inhibitors. T-cells derived from patients with PTEN deficient gliomas were stimulated and co-cultured with autologous glioma cells previously treated with or without interferon- γ (+ IFN) and/or with PI3K pathway inhibitors. Co-cultures were analyzed for T-cell apoptosis by analyzing % of target population (CD45+) that contained Annexin V staining. T-cells treated with staurosporin were used as positive control (not shown). Bars represent mean of triplicate samples from a representative experiment. Error bars represent $\pm$ SD. (* $P < 0.01$)