Induction of PD-L1 by Nitric Oxide via JNK Activation in A172 Glioblastoma Cells

Yoshimitsu Kiriyama*, Anna Tani, Minako Kadoya, Ryoko Okamoto, and Hiromi Nochi*

Kagawa School of Pharmaceutical Sciences, Tokushima Bunri University,
Shido 1314-1, Kagawa, Sanuki 769-2193, Japan

*Correspondence to: Yoshimitsu Kiriyama (Ph.D.) and Hiromi Nochi (Ph.D.),
Kagawa School of Pharmaceutical Sciences, Tokushima Bunri University,
Shido 1314-1, Kagawa, Sanuki 769-2193, Japan
E-mail: kiriyamay@kph.bunri-u.ac.jp (Y.K.) and nochi@kph.bunri-u.ac.jp (H.N.);
Abstract

Glioblastoma comprises 54% of all the gliomas derived from glial cells and are lethally malignant tumors of the central nervous system (CNS). Glioma cells disrupt the blood–brain barrier, leading to access of circulating immune cells to the CNS. Blocking the interaction between programmed cell death 1 (PD-1) and programmed cell death 1 ligand 1 (PD-L1) enhances T-cell responses against tumor cells, and inhibition of the PD-1/PD-L1 pathway is used as immunotherapy for cancer, including glioblastoma. Nitric oxide (NO) has multiple physiological roles, such as immune modulation and neural transmission in the CNS. Moreover, it has both tumor-promoting and tumor-suppressive functions. We examined the effects of NOC-18, an NO donor, on the expression of PD-L1 in A172 glioblastoma cells. NOC-18 increased PD-L1 expression in A172 glioblastoma cells. Moreover, this increase is regulated via the JNK pathway.

Key words c-jun N-terminal kinase; glioblastoma; nitric oxide; programmed cell death 1 ligand 1
INTRODUCTION

Glioblastoma comprises 54% of all the gliomas derived from glial cells of the central nervous system (CNS) in humans. Glioblastoma is a common and lethal primary malignant tumor. It is associated with strong aggressiveness and high mortality rates. Glioma cells disrupt the blood–brain barrier (BBB), leading to access of circulating immune cells to the CNS.

The interaction between programmed cell death 1 (PD-1), an immune checkpoint protein, and programmed cell death 1 ligand 1 (PD-L1), a ligand of PD-1, can result in inhibition of the T-cell response. Blocking the interaction between PD-1 and PD-L1 results in enhancement of T-cell responses against the tumor cells. Furthermore, inhibition of the PD-1/PD-L1 pathway can be used as immunotherapy for cancer, including glioblastoma.

Nitric oxide (NO) plays multiple distinct physiological roles, such as immune modulation and neural transmission in the CNS. Moreover, NO has both tumor-promoting and tumor-suppressive functions. In the CNS, it has been reported that blocking of PD-L1 results in a decrease in NO release from microglia. However, the effects of NO on the expression of PD-L1 in glioblastoma cells has not been elucidated yet. Therefore, we examined the effects of the long-acting NO donor NOC-18, also known as diethylenetriamine nitric oxide adduct (DETA/NO) or diethylenetriamine NONOate (DETA NONOate), on PD-L1 expression in A172 glioblastoma cells, which express PD-L1.
MATERIALS AND METHODS

Materials

NOC-18 was obtained from Dojindo (Kumamoto, Japan). SP600125, SB203580, and PD98059 were from Cayman Chemical (MI, USA). Anti-PD-L1 antibody was from ProSci (CA, USA). Anti-JNK antibody and anti-phospho-JNK (p-JNK) antibody were from Santa Cruz Biotechnology (CA, USA). Anti-tubulin antibody and horseradish peroxidase-linked secondary antibodies were from MBL (Nagoya, Japan).

Cell culture

The human glioblastoma cell line A172 was obtained from JCRB cell bank (Osaka, Japan). A172 cells were maintained in DMEM supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere with 5% CO₂.

Real-time quantitative PCR

Total RNA isolated from A172 cells was purified using MagExtractor RNA (TOYOBO, Osaka, Japan). Reverse transcriptase reactions were performed on 0.4 μg of RNA using a ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO, Osaka, Japan) in a 10 μl volume at 37°C for 15 min. Real-time quantitative PCR was performed with THUNDERBIRD SYBR qPCR Mix (TOYOBO, Osaka, Japan) and QuantStudio 7 (Thermo Fisher Scientific, Waltham, MA) using the following primers: PD-L1, 5’-GGCATTTGCTGAACGCAT-3’ and 5’-CAATTAGTGAGCCAGGT-3’; β2-microglobulin (B2M), 5’-ACTGAATTCAACCCACTGA-3’ and 5’-CCTCCATGATGCTGCTTACA-3’. Relative standard curves were generated using serial dilutions of cDNA to determine amplification efficiencies for each target gene. The expression level of a target gene was compared with the expression of B2M for each sample. Relative expression levels were calculated using REST software 12).
Western blot analysis

A172 cells were harvested and lysed in modified RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 2 mM diisopropylfluorophosphate, 10 µg/mL of leupeptin, 10 µg/mL of aprotinin, and 10 µg/mL of pepstatin) on ice for 20 min. Cell lysates were sonicated and centrifuged at 12,000 × g at 4°C for 10 min. Proteins in the supernatants were electrophoresed in SDS polyacrylamide gels and transferred to Immobilon P membranes (Millipore, Billerica, MA). The membranes were blocked with 1% skim milk in 0.1% Tween 20/PBS (PBS-T) and incubated with primary antibodies (dilutions based on manufacturer's recommendations). The membranes were washed three times in PBS-T and incubated for 1 h with a horseradish peroxidase-conjugated secondary antibody. Immunoreactive proteins were detected with EzWestLumi plus (ATTO Corp., Tokyo, Japan).

Statistical analysis

The data are expressed as the mean ± SEM of at least three independent experiments. Multiple comparisons were examined by ANOVA, followed by the Student's t-test. *p*<0.05 was considered statistically significant.
RESULTS AND DISCUSSION

The mRNA expression of PD-L1 in A172 glioblastoma cells was assessed by quantitative PCR. A172 glioblastoma cells were treated with the indicated concentrations of NOC-18 and total RNA was extracted after 24 h (Figure 1A). PD-L1 mRNA expression level significantly increased with 0.2 mM NOC-18, and the increase in PD-L1 mRNA expression reached a maximum (approximately three times that of the control) with 0.4 mM NOC-18. This expression was kept to 1 mM NOC-18. We also investigated the effects of 0.4 mM NOC-18 on the mRNA expression of PD-L1 at various time points up to 36 h (Figure 1B). The PD-L1 mRNA expression level was not significantly increased by 0.4 mM NOC-18 at up to 9 h. However, PD-L1 mRNA expression level increased significantly at 12 h and reached a maximum (approximately three times that of the control) at 24 h. This increased PD-L1 mRNA expression decreased at 36 h.

ERK and JNK are involved in the expression of PD-L1 \(^4, 13, 14\). Therefore, we then investigated the effects of SP600125, a JNK inhibitor, PD98059, a mitogen-activated protein kinase (MEK) inhibitor, and SB203580, a p38 MAPK inhibitor, on mRNA expression of PD-L1 induced by 0.4 mM NOC-18 for 24 h. A172 glioblastoma cells were pretreated with 3 or 10 \(\mu\)M of inhibitors or vehicle alone for 30 min, and were then incubated with a medium containing the same dose of inhibitors or vehicle alone, with or without 0.4 mM NOC-18, for 24 h. SP600125 decreased the PD-L1 mRNA expression induced by 0.4 mM NOC-18 (Figure 1C). However, PD98059 and SB203580 did not affect the PD-L1 mRNA expression induced by 0.4 mM NOC-18 (Figure 1D and 1E). Thus, PD-L1 mRNA expression by NO might be induced via the JNK activation in A172 glioblastoma cells.

We performed Western blot analysis to investigate the activation of JNK by quantification of the ratio of phospho-JNK (p-JNK) to JNK. A172 glioblastoma cells were treated with 0.4 mM NOC-18 at various time points up to 3 h and p-JNK/JNK ratio was calculated (Figure 2A). This ratio significantly increased with 0.4 mM NOC-18 at 30 min. We then investigated the effects of SP600125 on protein expression of PD-L1 treated with 0.4 mM NOC-18 for 24 h. A172 glioblastoma cells were pretreated with 3 or 10 \(\mu\)M of SP600125 or...
vehicle alone for 30 min, and were then incubated with a medium containing the same dose of SP600125 or vehicle alone, with or without 0.4 mM NOC-18, for 24 h. 0.4 mM NOC-18 increased PD-L1 protein expression and SP600125 decreased PD-L1 protein expression induced by 0.4 mM NOC-18 (Figure 2B). These results suggest that NO induces both PD-L1 mRNA and protein expression via JNK activation.

NO is generated from L-arginine by nitric oxide synthase (NOS) and corticosteroids inhibit the expression of NOS. Moreover, NOS is inhibited by many molecules including NOS dimerization inhibitors, such as BBS-2 and KLYP956, substrate competitive analogues, such as L-NAME and L-NIL, and non-amino acid derivatives, such as MEG and1400W. In addition, cell-free hemoglobin and carboxy-PTIO scavenges NO \(^{15-17}\). These molecules can inhibit the production or effects of NO.

**Conclusion**

In conclusion, we reported that NO induced the expression of PD-L1 in A172 glioblastoma cells and this induction was regulated by JNK activation. Inhibition of NO production or NO signaling can inhibit the PD-1/PD-L1 pathway by reducing the expression of PD-L1 and might function to suppress the proliferation of glioblastoma.

Conflict of Interest: The authors declare no conflict of interest.
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Fig. 1. Dose-dependent effects of NOC-18 on mRNA expression of PD-L1 in A172 glioblastoma cells. A172 glioblastoma cells were treated with the indicated concentrations of NOC-18 for 24 h (A). Time course of mRNA expression of PD-L1 with NOC-18 treatment in A172 glioblastoma cells. A172 glioblastoma cells were treated with 0.4 mM NOC-18 for the indicated times (B). The effects of SP600125 (C), PD98059 (D), and SB203580 (E) on mRNA expression of PD-L1 induced by 0.4 mM NOC-18 for 24 h are shown. A172 glioblastoma cells were pretreated with 3 or 10 μM MAPK inhibitors or vehicle alone for 30 min, and were then incubated with a medium containing the same dose of inhibitors or vehicle alone, with or without 0.4 mM NOC-18, for 24 h. The mRNA expression levels of PD-L1 were compared with those of B2M mRNA, which was used as an internal control. The ratios of treated to control levels (means ± SEM, n = 4) are shown. N.S.: not significant, *p < 0.05: control vs. NOC-18, #p < 0.05: NOC-18 without SP600125 vs. NOC-18 with SP600125.
Fig. 2. Western blot showing the effects of NOC-18 on the protein expression of phospho-JNK (p-JNK), JNK, and PD-L1. A172 glioblastoma cells were treated with 0.4 mM NOC-18 for the indicated times. Representative images show the bands for p-JNK and JNK with 0.4 mM NOC-18 for the indicated times. The ratio of p-JNK to JNK was determined by densitometry (means ± SEM, \( n = 4 \)); * \( p < 0.05 \) (A). A172 glioblastoma cells were pretreated with 3 or 10 \( \mu \)M SP600125 or vehicle alone for 30 min and incubated with medium containing the same dose of SP600125 or vehicle alone, with or without 0.4 mM NOC-18, for 24 h. Representative images show the bands for PD-L1. The ratio of PD-L1 to tubulin was determined by densitometry. Results are the ratios of treated to control levels (means ± SEM, \( n = 4 \)); * \( p < 0.05 \): control vs. NOC-18, \# \( p < 0.05 \): NOC-18 without SP600125 vs. NOC-18 with SP600125 (B).