International Immunopharmacology 54 (2018) 39–45

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Nafamostat mesilate, a serine protease inhibitor, suppresses interferon-gamma-induced up-regulation of programmed cell death ligand 1 in human cancer cells

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ARTICLE INFO

Keywords:
Nafamostat mesilate
Programmed cell death ligand-1
Interferon-gamma
Programmed cell death-1
Human leukocyte antigen class I
Lung cancer

ABSTRACT

Programmed cell death ligand-1 (PD-L1) plays a pivotal role in the suppression of antitumour immunity by binding to programmed cell death-1 (PD-1) on tumouricidal cytotoxic T lymphocytes (CTLs), rendering them inactive. As blockade of PD-1/PD-L1 interaction by the monoclonal antibodies induced effective T cell-mediated antitumour response, suppression of PD-L1 expression in tumour cells by the chemical agent might contribute to treatment against malignant tumours. Nafamostat mesilate (NM), a serine protease inhibitor that is frequently used in the clinic, potently suppressed interferon-gamma (IFN-gamma)-induced up-regulation of PD-L1 in cultured human lung cancer cells (HLC-1) at both the messenger RNA (mRNA) and protein levels. Interestingly, suppression of IFN-gamma-induced up-regulation of human leukocyte antigen (HLA)-ABC by NM was limited, suggesting that NM did not block CTL responses to tumour cells. NM treatment did not affect the activation status of signal transducer and activator of transcription (STAT) 1 or the induction of interferon regulatory factor (IRF)-1 expression in IFN-gamma-treated HLC-1 cells. Although NM treatment promoted the phosphorylation of extracellular signal-regulated kinases (Erk) 1/2, an Erk inhibitor, U0126, could not reverse the suppression of PD-L1 up-regulation by IFN-gamma. Suppression of IFN-gamma-induced up-regulation of PD-L1 by NM was not associated with the inhibition of nuclear factor kappa B (NF-*k*B) or protease-activated receptor (PAR)-1 pathway. Besides HLC-1 cells, NM suppressed IFN-gamma-induced PD-L1 up-regulation in three human pancreatic cancer cell lines. NM could potentiate the antitumour effect of cancer vaccines or immune checkpoint inhibitors by preventing IFN-gamma-induced PD-L1 up-regulation and blocking immune checkpoint suppression.

1. Introduction

Immune checkpoint blockade therapy has been shown to be efficacious in cancer therapy and has revolutionized conventional cancer treatments [1]. Currently, immune checkpoint blockade therapy is the leading choice for treatment of non-small cell lung cancers [2]. Monoclonal antibodies (mAbs) against the immune checkpoint-associated co-inhibitory molecules and their ligands have been used in innovative anti-cancer strategies [3]. Specifically, the anti-PD-1 monoclonal antibodies nivolumab and pembrolizumab counteract the suppression of antitumour immunity mediated by the PD-1/PD-L1 axis and reactivate the immune response to neo-antigens generated by genomic mutations in cancer cells [4,5]. These treatments result in significant and durable tumour regression for several malignancies. However, treatment using monoclonal antibodies for immune checkpoint blockade is expensive, and continuous treatment for long periods could result in high-cost medical care. Accordingly, immune checkpoint blockade therapy using agents other than monoclonal antibodies is now being explored.

Anti-PD-1 monoclonal antibodies, which are relatively safer and more effective than the anti-CTLA-4 mAb [6], are commonly used to treat various malignancies [7]. PD-L1, a ligand for PD-1, is expressed on target tumour cells as well as antigen-presenting cells [8] and inhibits the induction and function of T cell-mediated antitumour immunity. PD-L1 expression is constitutive or inductive, and IFN-gamma induces PD-L1 expression [9]. Blockade of the interaction between PD-1 on IFN-

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http://dx.doi.org/10.1016/j.intimp.2017.10.016
Received 17 May 2017; Received in revised form 10 October 2017; Accepted 16 October 2017
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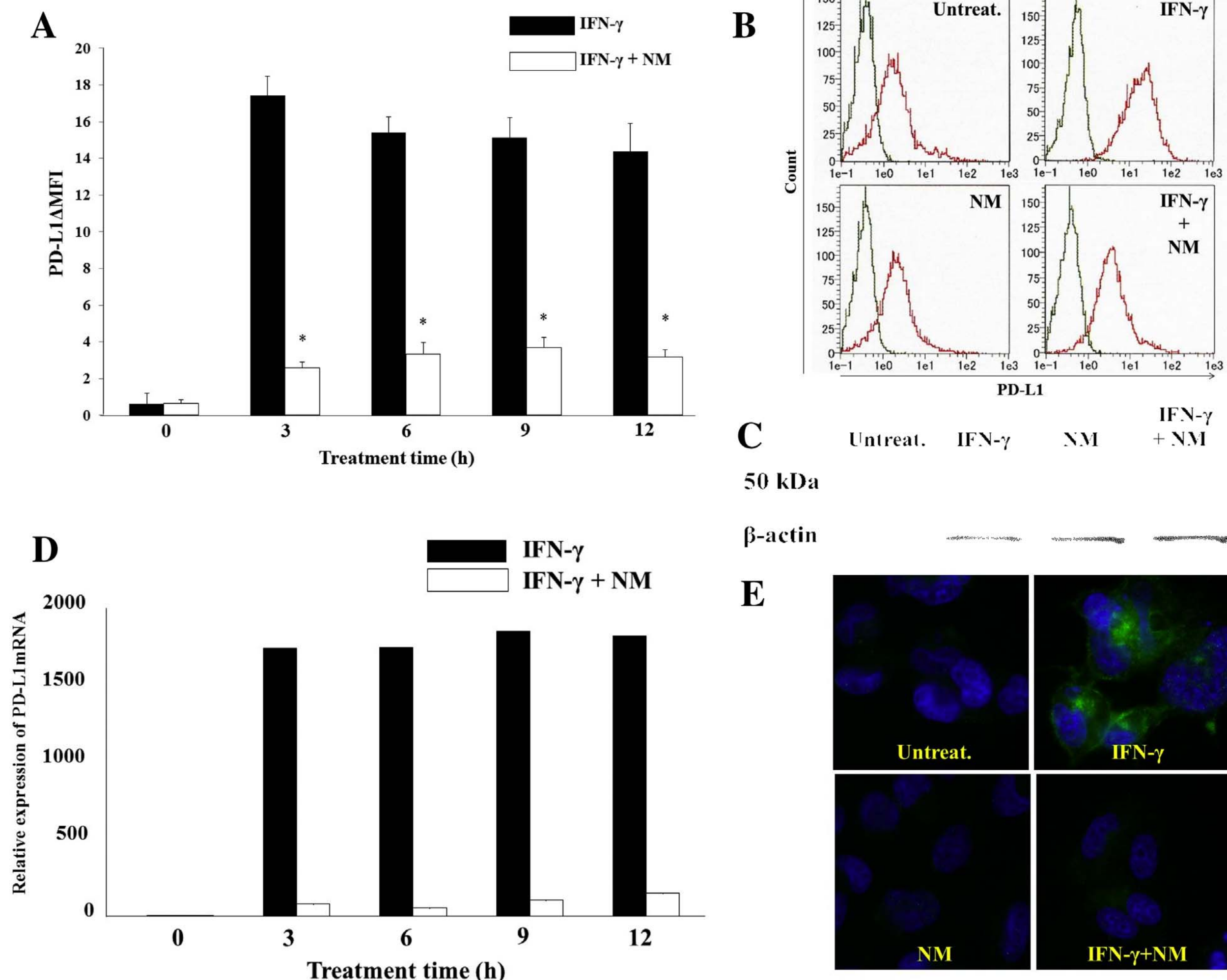

Fig. 1. NM suppressed IFN-gamma-induced PD-L1 up-regulation in HLC-1 cells. A; HLC-1 cells were treated with recombinant human IFN-gamma (100 ng/ml) and/or NM (100 μg/ml) for 3, 6, 9 or 12 h and followed by another incubation in fresh medium for 45, 42, 39 and 36 h, respectively. PD-L1 expression was examined by flow cytometry after total 48 h incubation (n = 3). PD-L1 ΔMFI shows the MFI of PD-L1 minus the MFI of the isotype control. This study was repeated three times with similar results. *$p < 0.01$. B; HLC-1 cells were treated with IFN-gamma (30 ng/ml) and/or NM (100 μg/ml) for 3 h. After another 45 h incubation in the fresh medium, PD-L1 expression was examined by flow cytometry. The green and red histogram profiles indicate the isotype control and PD-L1 expression, respectively. C; HLC-1 cells were treated with IFN-gamma (30 ng/ml) and/or NM (100 μg/ml) for 3 h. After another 45 h incubation in the fresh medium, PD-L1 expression was examined by western blot analysis. D; HLC-1 cells were treated with IFN-gamma (30 ng/ml) and/or NM (100 μg/ml) for 3 h. PD-L1 mRNA expression was examined by real-time PCR after another 45 h incubation in the fresh medium (n = 3). Relative expression was calculated using the delta-Ct method. This study was repeated three times with similar results. E; HLC-1 cells were treated with IFN-gamma (30 ng/ml) and/or NM (100 μg/ml) for 3 h. After another 45 h incubation in fresh medium, PD-L1 expression was examined by immunofluorescence microscopy. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

gamma-producing CTLs and PD-L1 induced by IFN-gamma on tumour cells is important to activate tumouricidal CTLs because a strong intrinsic immune response to tumour neo-antigens might be inhibited by the PD-1/PD-L1 axis [10]. In fact, PD-L1 can be visualized by immunohistochemical analysis in T cell-inflamed areas of tumour tissues [11], indicating that PD-L1 is induced by IFN-gamma produced by antitumour T cell responses. This inducible PD-L1 expression renders PD-1$^+$ CTLs incompetent. Constitutive PD-L1 expression in tumour tissues is caused by uncontrolled activation of oncogenic cell signalling [12]. Loss of phosphatase and tensin homologue (pten) expression leads to oncogenic activation of the phosphoinositide 3-kinase (PI3K) pathway, which is closely associated with constitutive PD-L1 expression in tumour cells [13,14]. However, the significance of constitutive PD-L1 expression in tumour tissues and its association with immune suppression is unclear.

Recently, mAb-independent immune checkpoint blockade therapy was explored. Suppression of IFN-gamma-inducible PD-L1 expression on tumour cells may be beneficial for immune checkpoint blockade, as PD-L1 induced by IFN-gamma inactivates PD-1$^+$ CTLs by binding to PD-1. Although some studies examining the suppression of IFN-gamma-inducible PD-L1 expression have been reported, this strategy is difficult to apply in practical clinical cancer therapy [15,16].

The expression and activity of cell surface proteases increase during carcinogenesis, and the proteases expressed in cancer cells are associated with malignant phenotypes, such as vigorous proliferation, invasion and metastasis [17,18]. Accordingly, inhibition of cancer cell proteases should alter the biological behaviours of cancer cells. Nafamostat mesilate (NM) has a broad spectrum as a serine protease inhibitor and has been used for the treatment of pancreatitis [19,20]. Additionally, NM exhibits antitumour activity by modulating cancer cell physiology [21]. Anti-proliferative effects, inhibition of cell adhesion and invasion, and increased anoikis sensitivity were observed in cancer cells following NM treatment [22]. Furthermore, inhibition of ICAM-1 and VEGF expression and suppression of matrix metalloproteinase-2 and 9 activities may contribute to the anticancer effects of NM [22]. Suppression of nuclear factor kappa B (NF-*k*B) activation by NM is closely associated with the modulation of biological activity and resistance to chemotherapeutic agents [22–24]. Combined treatment with

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