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Original Article

HMGB1 and HMGB2 Effects on Programmed Cell Death Ligand 1 With the Receptor for Advanced Glycation-End Product Pathway in the Monocyte Cells of Patients With Multiple Sclerosis

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Abstract

Background: High-mobility group box 1 (HMGB1) is a nonhistone, DNA-binding protein that serves a crucial role in regulating gene transcription and is involved in various proinflammatory, extracellular activities. The aim of this study was to explore whether HMGB1 stimulation can upregulate the expression of programmed cell death ligand 1 (PD-L1).

Methods: Thirty multiple sclerosis (MS) patients at mild, moderate, and severe stages were recruited from the MS clinic. Blood samples were collected from patients, and monocyte cells were isolated from peripheral blood mononuclear cells (PBMCs) using magnetic-activated cell sorting brush separation. The isolated monocytes were cultured and stimulated with HMGB1 and HMGB2 over a time course of 0 hour, 6 hours, 12 hours, 24 hours, and 48 hours.

Results: The effects of HMGB1 and HMGB2 stimulation were evaluated over time. PD-L1 expression increased significantly throughout the time course, with peak increases observed for HMGB1 and HMGB2 24 and 12 hours after stimulation, respectively.

Conclusion: Evidence from the present study suggests that enhanced PD-L1 expression may result from HMGB1 and HMGB2 stimulation in MS patients.

Keywords: HMGB1, HMGB2, MS

Introduction

Multiple sclerosis (MS) is a neurological disorder characterized by autoimmune demyelination of the nervous system, affecting approximately 2.3 million people's lives worldwide.[1,2] The development of MS is attributed to a complex interplay of genetic predisposition, epigenetic factors, lifestyle choices, and environmental exposures. However, the autoimmune nature of the disease clearly indicates the major involvement of the immune system. MS involves both innate and adaptive immune mechanisms, with inflammation playing a crucial role through a diverse array of immune cells.[3]

Programmed cell death protein 1 (PD-1) is an inhibitory co-receptor belonging to the CD28 family, primarily expressed on T cells and B cells. Programmed cell death ligand 1 (PD-L1), the main ligand for PD-1, is a member of the B7 family and is expressed on various immune cells, including monocytes and macrophages.[4] The PD-1/PD-L1 binding creates an inhibitory axis that provides important regulatory signals.[5,6] This axis has been extensively studied in cancer research and has demonstrated a major role in tumor growth.[7] Additionally, research has confirmed its essential role in immune self-tolerance.[8]

Studies have revealed reduced levels of PD-1 and PD-L1 molecules in the blood serum of patients with MS disease.[9] Comparatively, individuals with relapsing-remitting MS exhibit significantly lower levels of PD-1 and PD-L1 expression in peripheral blood mononuclear cells (PBMCs) compared to healthy individuals, indicating a disruption of immune tolerance in MS [3]. Additionally, the analysis of cerebrospinal fluid (CSF) in MS patients has shown a modest age-associated increase of PD-L1 in CSF.[10-15]

High-mobility group (HMG) proteins constitute a widespread superfamily of nuclear proteins that interact with DNA and nucleosomes, altering the structure of chromatin fibers. These proteins play significant roles in chromatin dynamics and influence DNA processing within the chromatin context, forming a specialized chromatin structure called the enhanceosome. HMGA and HMGB proteins represent two distinct families within this category. Many studies have investigated both groups in the oncology field.[16] *HMGA2*, a member of this family, resides on chromosome 12q14-15. This gene exhibits

elevated expression in pluripotent embryonic stem cells during embryogenesis but remains minimally expressed or absent in adult tissues.[15,17] However, a notable exception is its re-expression in various human malignancies.[18] The expression and function of *HMGA2* have been extensively studied in multiple cancers.[19]

HMGB proteins play a crucial role in orchestrating several essential genomic processes, including DNA repair, nucleosome movement, telomere maintenance, and transcription. Their regulation of these fundamental cellular processes not only supports normal cellular functions but also exerts a profound impact on a diverse range of disease states, including cancers and autoimmune disorders. High-mobility group box 1 (*HMGB1*) and high-mobility group box 2 (*HMGB2*) are the two members of this family.[20] *HMGB2* is predominantly expressed in immune cells.[21] Studies have demonstrated that *HMGB2* can trigger harmful processes, such as increased reactive oxygen species production, inflammation, apoptosis, and impaired autophagosome clearance. A well-established correlation in this context is the effect of HMGB2 on ischemic heart disease.[22] However, there is a lack of information regarding the possible role of HMGB2 in MS or other autoimmune diseases. In contrast, HMGB1, which is highly homologous to HMGB2, has been studied in the context of MS disease. Elevated levels of extracellular HMGB1 and its receptors, the receptor for advanced glycation end products (RAGE) and Toll-like receptors, have been detected in the plasma and CSF of MS patients.[23,24]

The RAGE, discovered in 1992, is an immunoglobulin superfamily member with various extracellular ligands. It is involved in the development of various inflammatory disorders and is thought to activate multiple intracellular signaling pathways that contribute to chronic inflammation and promote malignant transformation. Interactions through RAGE have been associated with increased cell proliferation, metastasis, and reduced apoptosis. RAGE is overexpressed in many types of cancer, and elevated levels have been associated with poor cancer outcomes.[25] RAGE was the first receptor identified to bind HMGB1 and initiate its biological effects. HMGB2 also interacts with RAGE.[26]

Fingolimod, a medication used to treat MS, appears to modulate the RAGE axis. This modulation may contribute to fingolimod's anti-inflammatory and neuroprotective effects, suggesting potential efficacy in other pathological conditions where RAGE dysregulation is involved. Studies have shown a decrease in serum HMGB1 levels with fingolimod treatment.[27] Additional research has confirmed that blocking RAGE signaling with antibodies (RAGE Ab) significantly reduces both HMGB2 and HMGB1 levels.[28]

Phosphoinositide 3-kinases (PI3Ks) are a family of enzymes involved in cellular functions. According to research, these molecules are found in association with HMG molecules in various conditions characterized by immune system dysfunction. The PI3K/protein kinase B (AKT) pathway, which is involved in learning and memory, is significantly decreased in mice with Alzheimer's disease (AD). HMGA2, which regulates the expression of the PI3K/AKT pathway, may play a negative role in AD. Silencing HMGA2 in AD mice resulted in the increased expression of the PI3K/AKT pathway and improved their condition.[29] Additionally, targeting HMGA2 and blocking the PI3K/AKT signaling pathways suppressed the progression of hepatocellular carcinoma.[30] The RAGE/PI3K signaling pathway, in conjunction with other molecules, has been associated with various pathologies. For example, the overexpression of RAGE inhibited cell apoptosis by reducing the ratio of *Bax* to *Bcl-2* and activating the PI3K/AKT signaling pathway in cervical squamous cancer.[25] This pathway was also involved in breast cancer.[31]

The HMG family, PD-1/PD-L1 axis, and the RAGE/PI3K signaling pathway have been extensively studied in cancer research. Given that sufficient data demonstrate the presence of these elements in autoimmune disorders and the similarities in immune system involvement between cancer and autoimmune pathogenesis mechanisms, there is considerable potential for more comprehensive studies in autoimmunity.

Previous studies have confirmed the role of monocytes in MS pathogenesis.[32] The present study aims to investigate the potential effects of HMGA2 and HMGB2 molecules through the RAGE/PI3K signaling pathway on PD-L1 expression in monocytes isolated from MS patients.

Materials and Methods

Peripheral Blood Mononuclear Cells and Cell Culture

In this case-control study, blood samples were collected from 30 MS patients (age range: 17–40 years) at mild, moderate, and severe disease stages referred to the MS clinic. Monocytes were isolated from PBMCs using magnetic-activated cell sorting separation. The inclusion criteria were MS patients under treatment for at least two years. On the other hand, the exclusion criteria included pregnancy, treatment discontinuation, recent diagnosis, and vulnerable individual status.

Monocytes were isolated and cultured using the magnetic-activated cell sorting method. Heparinized blood was diluted with 10 mL of Dulbecco's modified Eagle culture medium (DMEM). The diluted blood was carefully layered over an equal volume of Ficoll and centrifuged at 800 g for 15 minutes. The isolated cells were resuspended in DMEM and centrifuged at 200 g for 10 minutes to remove platelets along with PBMCs. In addition, cell count and viability were determined using Trypan Blue staining. For each flask, 1×10^7 cells were cultured in DMEM supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% serum, with one flask serving as a control. CD14 expression levels were assessed to confirm monocyte purity using the MACS method. The cells were treated with 10 µL of

HMGB1 and HMGB2 for 2 hours, 6 hours, 12 hours, 24 hours, and 48 hours.

Real-Time Polymerase Chain Reaction

All RT-PCRs were performed using the Rotor Gene™ 6000 (Corbett). The thermal cycling program consisted of initial denaturation at 95 ˚C for 5 minutes, 40 cycles of denaturation at 95 ˚C for 10 seconds, annealing at 56 ˚C for 10 seconds, and extension at 60 ˚C for 24 seconds, and a final melting curve analysis with temperature increasing from 50˚C to 99 ˚C at 1 ˚C/5 seconds.

Reactions were performed in duplicate using 0.1 mL microtubes with a final volume of 10 µL, containing 5 µL of 2X QuantiFast SYBR Green PCR Master Mix, 0.5 µL of each primer (10 pmol), 2 µL of ribonuclease-free water, and 2 µL of template complementary DNA. Standard curves were generated using serial dilutions (51–55) of control complementary DNA. PCR efficiency was determined from the standard curve for each gene. A no-template control was included as a negative control.

Western Blot

HMGB1-treated and HMGB2-treated monocytes were lysed and separated on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis gels before transfer to polyscreen polyvinylidene fluoride membranes (PerkinElmer, USA). The membranes were blocked with 5% (w/v) non-fat dry milk and 1% (v/v) Tween 20 in phosphate-buffered saline for 1 hour at room temperature and then incubated overnight with the anti-RAGE antibody (1:1000) (Abcam, USA) at 4˚C. The protein was detected using electrochemiluminescence, and the blots were quantified by densitometry using image analysis software (Amercontrol Biosciences, USA).

Statistical Analysis

Statistical analyses were performed using software, version 17.0. The obtained data are presented as means ± standard deviations. Between-group comparisons were conducted using t-tests or one-way analysis of variance with Bonferroni correction. Statistical significance was set at $P < 0.05$. Data visualization was performed using Corbett RT-PCR, FACS, and SPSS software packages.

Results

Increased Expression of Programmed Cell Death Ligand 1 by High-Mobility Group Box 1 and High-Mobility Group Box 2 Stimulation

This study evaluated the effects of HMGB1 and HMGB2 stimulation over time on PD-L1 expression. PD-L1 expression increased significantly with time, showing peak increases for HMGB1 and HMGB2 after 24 hours and 12 hours of stimulation. β-actin served as an internal control in these experiments (Figure 1).

Enhanced Receptor for Advanced Glycation-End Product Pathway Activation by High-Mobility Group Box 1 and High-Mobility Group Box 2 Stimulation

Western blot analysis demonstrated enhanced RAGE expression in monocytes from MS patients following HMGB1 and HMGB2 stimulation. This enhancement was particularly pronounced following HMGB1 stimulation at 4 hours and 8 hours. Figure 2 displays a representative Western blot with β-actin as the loading control.

Discussion

In MS disease, so far, the results and effectiveness of treatments have not been satisfactory. In addition, many of these treatments have harmful side effects that are difficult for patients to tolerate. Based on the history of MS, this disease was treated by different doctors in different ways about a century ago.[8] Perhaps the most important reason for these different treatments was the doctors' thinking about the cause of the disease. However, in the 20th century, it was determined that MS is an autoimmune disease, and research on this disease took a new direction accordingly. The body's immune system has a special complexity with various components and structures. The function of these components, when faced with a specific situation, requires precise regulation and development within the immune system. Some studies have shown that in MS, although the number of T lymphocyte cells is normal, they are functionally defective, and in MS patients, regulatory T cells have less power to suppress interleukin 17 production compared to healthy people.[26] Some studies demonstrated a decrease in the number of immune cells in MS patients.[6] In addition, it has been reported that

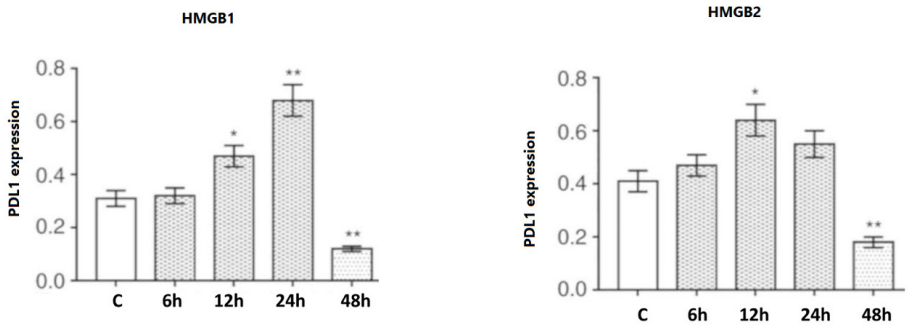

Figure 1. The Expression of PD-L1 With HMGB1 and HMGB2 Stimulation. *Note*. PD-1: Programmed Cell Death Ligand 1; HMGB1: High-mobility group box 1; HMGB2: High-mobility group box 2

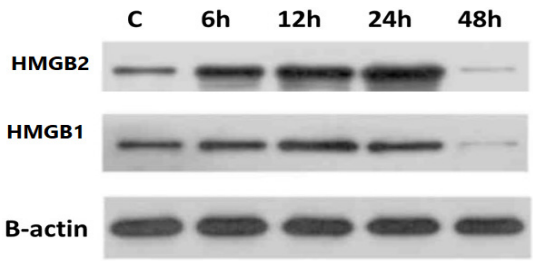

Figure 2. The Expression of the RAGE Pathway With HMGB1 and HMGB2. *Note*. RAGE: Receptor for advanced glycation end products; HMGB1: High-mobility group box 1; HMGB2: High-mobility group box 2

pro-inflammatory cytokines produced by regulatory T cells inhibit experimental autoimmune encephalomyelitis by inhibiting the response of autoreactive T cells in the central nervous system, and transforming growth factor-β inhibits the production of interleukin 17 and increases the expression of Foxp3 in T helper cells.[26] Zheng et al found that the regulatory function of innate immune cells in patients with MS is defective, and the lack of regulatory function of these cells plays an essential role in the pathogenesis of this disease.[26] The role of PD-1 and PDL-1 molecules on the immune cells of MS patients has been a topic of interest in the literature in recent years. It has been shown that in response to neuroinflammation, microglia, a type of immune cell in the central nervous system, show enhanced expression of both PD-1 and PD-L1.[11] Additionally, numerous studies indicated that T cells interacting with the PD-1/PD-L1 pathway play a crucial role in the development of MS.[12] Other papers revealed a correlation between polymorphism in PD-1 and the prognosis of MS.[13,14] Trabattoni et al defined specificity in PD-1 and PDL-1 expressions in different patterns of the disease.[15] Some findings suggest that blocking PD-1 signaling could reduce inflammation and improve the clinical course of the disease, and thus could potentially be a therapeutic target.[11]

Conclusion

In our study, by extracting monocyte cells from blood and cultivating them as macrophages, the findings revealed that this gene can lead to the growth and increased activity of macrophage cells, which are pro-inflammatory cells.

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Authors' Contribution

Conceptualization: Ilgar Amjadi, Ramin Ahangar.
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Visualization: Ilgar Amjadi, Ramin Ahangar.
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Competing Interests

None to be declared.

Ethical Approval

This study was approved by Research Ethics Committees of Tabriz University of Medical Sciences(Ethical code: IR.TBZMED.REC.1402.547).

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