G2013 modulates TLR4 signaling pathway in IRAK-1 and TARF-6 dependent and miR-146a independent manner

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Abstract: Inflammation is an inseparable part of different diseases especially cancer and autoimmunity. During inflammation process toll like receptor 4 (TLR4) responds to lipopolysaccharide (LPS), one of the bacterial components, and TLR4 signaling leads to interleukine-1 receptor associated kinase-1 (IRAK1) and tumor necrosis factor (TNF) receptor associated factor6 (TRAF6) activation which ultimately results in nuclear factor-κB (NF-κB) activation as the main transcription factor of inflammatory cytokines. Conversely, NF-κB over activation induces miR-146a in innate immune cells which can consequently reduce TRAF6, IRAK1, and NF-κB activation in a negative feedback. G2013 is a novel designed non-steroidal anti-inflammatory drug (NSAID) which was recently shown to be effective in experimental autoimmune encephalomyelitis (EAE) mouse model. The aim of this study was to evaluate G2013 effects on inflammatory (IRAK1 and TRAF6) and anti-inflammatory (miR-146a) factors of TLR4 signaling pathway. For this purpose, cytotoxicity of G2013 has been evaluated by MTT assay. Expression level of miR-146a in PBMCs and IRAK1 along with TRAF6 in HEK-293 TLR4 cells have been determined using real time PCR. Our results showed that IC50 of G2013 was 25μg/ml, thus 5 and 25 μg/ml concentrations used for further treatments as low dose and high dose concentrations. Our results showed that IRAK1 expression reduced between 5 to 8 fold after treatment by G2013 in a dose dependent manner (p<0.001). In parallel TRAF6 expression declined between 3 to 10 fold dose dependently (p<0.05). However, miR-146a expression was not affected after treatment with low dose and high dose of G2013. In conclusion our data showed that G2013 can regulate TLR4 signaling pathway during inflammation by reducing downstream signaling molecules, IRAK1 and TRAF6 without altering miR-146a expression.

Key words: miRNA, G2013, TLR, IRAK1, TRAF6, Inflammation.

Introduction

Innate immunity is considered as the first line of defense against exposure to pathogenic microorganisms. Lipopolysaccharid (LPS) is one of the bacterial components that can be recognized by innate immunity receptors especially toll like receptors (TLRs) (1). Among TLRs, TLR4 responds specifically to LPS and has the leading role in the initiation of inflammatory related signaling pathways. During TLR4 signal transduction, interleukine-1 receptor associated kinase-1 (IRAK1) and tumor necrosis factor (TNF) receptor associated factor 6 (TRAF6) are the main actors of downstream signaling events leading to nuclear factor-κB (NF-κB) activation as the main transcription factor of inflammatory cytokines (2).

Inflammation is undeniable part of different kinds of human diseases including autoimmunity, cancers and pathogen related illnesses. Signaling of TLRs as a part of innate immunity mainly results in generation of inflammatory cytokines causing the irritable symptoms of most of these diseases. Although until now there have been many approved anti-inflammatory medications such as non-steroidal anti-inflammatory drugs (NSAIDs), each of which have their own limitations including high toxicity and low effectiveness during application. Therefore it seems that developing new therapeutics with lower toxicity and higher efficacy is a global need (3, 4).

G2013, a novel NSAID with low molecular weight produced in immunology section of Pathobiology Department of Tehran University of Medical Sciences (TUMS) is an epimer of M2000 (5, 6). M2000 structurally is a β-D-Mannuronic acid derives from alginate and its anti-inflammatory effects have been previously investigated in different animal models (7–9). Hopefull results from experimental autoimmune encephalomyelitis mice treated with this new therapeutic agent, make G2013 a new candidate for anti-inflammatory therapy (10).

MicroRNAs(miRs) can be defined as a class of small, non-coding endogenous RNAs (18-25 nucleotides) that can regulate gene expression post transcriptionally (11). MiRNAs regulatory impressions are handled by recognizing their target mRNA by means of 5′-seed sequence and consequently interacting with the related miRNA regulatory elements located in 3′-untranslated region of the mRNA(12). MiRNAs play essential roles in managing different cell processes including cell proliferation, apoptosis and differentiation. Some of the identified
miRNAs that can act on cells of innate and adaptive immune system selectively change their expression rate according to different types of inciters such as bacterial components and pharmacological substances. Taking together, it has to be noted that miRNA changing levels correlate with the strength of immune system (13).

MiR-146a is one of the immune system regulators which its leading role has been perfectly identified in response to inflammatory cytokines and/or pathogen derived products such as LPS in many cells. Generally, during production of NF-κB, miR-146a transcriptionally induced as an inflammatory response by innate immune signaling (14, 15). Surprisingly induction of miR-146a in innate immune cells can consequently reduce TRAF6 and IRAK1 adaptor molecules that can finally inhibit NF-κB activation (16, 17). Here our approach was to determine the effects of G2013 on the expression levels of anti-inflammatory (miR-146a) and inflammatory (IRAK1 and TRAF6) factors of TLR4 signaling pathway.

Materials and Methods

Sampling

Blood samples were collected from healthy donors according to Tabriz University of Medical Sciences review board approval. Written consent letter was obtained from each donor. Peripheral blood mononuclear cells (PBMCs) were isolated by Histopaque (Sigma, ST Louis, USA) density-gradient centrifugation.

Cell culture

HEK 293-TLR4 cells were purchased from the cell bank of Pasteur institute (Tehran, Iran). Cells and isolated PBMCs were grown at 37 °C and 5% CO₂ pressure in RPMI 1640 (Gibco BRL, USA) supplemented with 10% heat-inactivated fetal cow serum (FCS) (Gibco BRL, USA), penicillin 100 units/ml and streptomycin 100 μg/ml (Gibco BRL, USA).

MTT cell proliferation assay

HEK 293-TLR4 cells were seeded at a density of 1.5x10³ cells/well in 96-well tissue culture plate, then media was replaced with 200 μl fresh media containing different concentrations of G2013 (2.5, 5, 25, 50, 100, 200 and 400 μg/ml) and incubated for 24, 48 and 72 hrs at 37 °C and 5% CO₂. The last row was defined as untreated control. After that the media was removed and the cells were washed by phosphate buffer saline (PBS), 200μl of 5mg/ml MTT solution was added to each well and incubated for 4hrs at 37 °C with 5% CO₂. Later 150 μl of MTT solution was aspirated off and 150μl DMSO was replaced to dissolve formazan crystals. Finally the plates were read at 570 nm in a micro titer plate reader. The same procedure was done for various concentrations of LPS (25, 50, 100, 200, 400, 800 and 1000 ng/ml) at 24hrs incubation time according to manufacturer’s instructions.

Cell Treatment

HEK-TLR4 cells and PBMCs were seeded in 6-well tissue culture plate at a density of 5x10⁵. 5 and 25 μg/ml concentrations were selected for cell treatment in 48hrs. In order to stimulate TLR4 signaling pathway a group of cells were also pre-stimulated with 100ng/ml of LPS for 24hrs (Invivogen, USA) according to manufacturer’s instructions. LPS stimulated cells were treated with the estimated drug doses for 48 hrs.

RNA extraction and Quantitative real time PCR

RNA extraction and real time PCR was done as previously described (18). Briefly, Total RNA was extracted by miReasy Mini Kit(Qiagen, Germany) and converted to c-DNA by Moloney murine Leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA, USA). The PCR product then subjected to SYBER green real time PCR (Takara, Japan). For analysis of miR-146a, extracted total RNA converted to cDNA using Mystiq microRNA cDNA Synthesis Mix (Sigma, USA). cDNA served as the template for real-time PCR analysis using the miScript Primer Assay (Qiagen, Germany) in combination with the SYBER green (Takara, Japan). Relative mRNA expression levels of the gene of interest were assessed by threshold cycle (Ct) based on ΔΔCt method. β-actin and U6 were chosen for mRNA and miRNA normalization, respectively. Each assay condition was measured in triplicates and results were averaged from at least three independent experiments. The sequences of the primers are mentioned in table1.

Table 1. Primers sequences.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>sequence</th>
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<tbody>
<tr>
<td>IRAK1F</td>
<td>5'-TGAAGAGGCTGAAGGAGAAG-3'</td>
</tr>
<tr>
<td>IRAK1R</td>
<td>5'-CGTACACCAGGCAGTAGAAG-3'</td>
</tr>
<tr>
<td>TRAF6F</td>
<td>5'-TCCGGTTAAGAAGAAGAAGG-3'</td>
</tr>
<tr>
<td>TRAF6R</td>
<td>5'-ATCAGAGAAGAGATGCCCTAATC-3'</td>
</tr>
<tr>
<td>B-Actin F</td>
<td>5'-AGCGCTCCCTCTGCCGTCG-3'</td>
</tr>
<tr>
<td>B-Actin R</td>
<td>5'-AGCCTGTGTTGGCGTGCTACAGGTC-3'</td>
</tr>
<tr>
<td>mir-146a F</td>
<td>5'-UGAGAACUGAAUUCGAUGGGUU-3'</td>
</tr>
<tr>
<td>Hsa-RNU6</td>
<td>5'-GUTTCCGACAGCUTATACATUAAT-3'</td>
</tr>
<tr>
<td>Universal primer</td>
<td>5'-UGAGAACUGAAUUCGAUGGGUU-3'</td>
</tr>
</tbody>
</table>

Statistical analysis

Data were expressed as mean ± SD using Graph pad prism 6 software and one-way ANOVA test for statistical analysis. P-values below 0.05 were regarded as statistically significant.

Results

G2013 acts time/dose dependently

HEK 293-TLR4 cells were exposed to G2013 at the concentrations of 0, 2.5, 5, 25,100,200 and 400 μg/ml for 24, 48 and 72 hrs and its cytotoxicity was assessed by MTT assay. Cytotoxicity was observed in dose and time dependent manner. G2013 IC50 was 25 μg/ml after 48hrs treatment (p<0.05)(Figure1a). In addition HEK293-TLR4 24hrs treatment with spectrum of LPS concentrations revealed no cytotoxicity even at high concentration of LPS (1000 ng/ml) (Figure1b).

MiR-146a expression level did not change significantly

In this study we used quantitative real-time PCR to analyze the changes in miR-146a expression. Our re-
G2013, the new modulator of TLR4 pathway.

M. Hajivalili et al. 2016 | Volume 62 | Issue 4

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results showed that G2013 did not significantly change miR-146a expression rate in treated PBMCs at 5 and 25 μg/ml concentrations in presence and absence of 100 ng/ml LPS. MiR-146a expression changed 1.25 fold (0.8±0.12, p>0.05) by 5μg/ml, 1.5 fold (0.66±0.07, p>0.05) after 25 μg/ml treatment in absence of LPS 100ng/ml. After co treatment of LPS with 5 and 25 μg/ml of G2103 the miR-146a altered expression was 1.21 (0.82±0.2, p>0.05) and 1.35 fold (0.73±0.03, p>0.05) which were not statistically significant. It is necessary to mention that miR-146a induced after treatment with LPS 100ng/ml (1.16 fold) (1.16±0.04, p>0.05) (Figure2).

G2013 down regulated gene expression of IRAK1 ad TRAF6

In this study mRNA expression levels of miR-146a target genes were evaluated after 48hrs exposure of HEK-293TLR4 cells with 5 and 25 μg/ml concentrations along with or without 100ng/ml of LPS. The results showed that G2013 could significantly reduce the mRNA levels of IRAK1 and TRAF6. The mRNA levels of IRAK1 were reduced to 4.34(0.23±0.12, p<0.001), 5.89(0.16±0.11, p<0.001), 7.14(0.14±0.09, p<0.001) and 7.69(0.13±0.04, p<0.001) by 5 and 25 μg/ml with and without LPS treatment, respectively. In high dose treatments expression of IRAK1 exhibited lower expression than low dose also this pattern was repeated in presence of LPS (Figure3a). Furthermore we assessed the effects of G2013 on the expression rate of TRAF6. In a similar way TRAF6 expression reduced 5.88(0.17±0.2, p<0.05), 12.5(0.08±0.03, p<0.05), 3.22(0.31±0.04, p<0.05) and 10(0.1±0.08, p<0.05) fold consequently after treatment with low dose and high dose. (p<0.05).

Figure1. HEK-293 TLR4 proliferation determined by MTT. HEK-293 cells were treated with different doses of G2013 for 24, 48 and 72 hrs separately. According to the results 5 μg/ml and 25 μg/ml were considered for low dose and high dose treatments. As represented in figure1b, HEK-293 TLR4 cells viability was not affected with various doses of LPS (25, 50, 100, 200, 400, 800, 1000ng/ml).

Figure2. G2013 reduced miR-146a expression level in treated PBMCs. RNA extracted from PBMCs treated under different conditions by LPS (100ng/ml), 5 and 25 μg/ml of G2013 and relative expression of miR-146a analyzed by qRT-PCR by use of U6 as internal control. G2013 did not affect the expression rate after treatment with low dose and high dose. (p>0.05).

Figure3. G2013 could significantly reduce the mRNA expression rate of IRAK1 and TRAF6. RNA of treated HEK293 TLR4 by G2013 and LPS under mentioned conditions was isolated and the expression level of IRAK1 and TRAF6 was assessed after qRT-PCR, in addition βactin used for normalizing. G2013 markedly reduced IRAK1(figure3a) and TRAF6(figure3b) expression ratio after each treatment in comparison to untreated cells in a dose dependent manner. *p<0.05, **p<0.001.
Both IRAK1 and TRAF6 significantly upregulated to 1.95(1.95±0.04, p<0.001) and 1.95(1.95±0.2, p<0.05) fold respectively after LPS treatment.

Discussion

In this study we revealed three main findings showing the function of G2013 in controlling inflammation by changing miR-146a, IRAK1 and TRAF6 expression. IRAK1 expression significantly reduced after treatment with low dose and high dose of G2013 by a dose dependent pattern in absence and presence of LPS. TRAF6 gene expression level significantly decreased by G2013 in absence and presence of LPS. Finally G2013 exerted its function without altering miR-146 expression.

Moreover we have demonstrated the effects of G2013, the novel designed NSAID, molecular mechanisms and uncovering new target molecules for G2013 in directing overstimulation of innate immunity responses.

MiR-146a is one of the posttranscriptional managers of immune cell signaling via TLRs especially TLR4. Normally, TLR4 activation by LPS stimulate NF-κB generation which is the major cause of miR-146a expression that lead to reduction of IRAK1 and TRAF6 expression respectively(17). Unregulated TLR or IL-1R activation is one of the pathological situations which vary from different types of inflammation to the onset of autoimmune diseases. There have been many efforts for modulating TLR/IL-1R responses, including direct blocking of receptor activation or inhibiting downstream signaling pathways (19). G2013 is an epimer form of M2000 (β- Gulluronic acid), the novel NSAID which its therapeutic efficacy has been reported in EAE previously (7). In 2013 treatment of EAE mouse models with α4- Gulluronic acid revealed histological and immunomodulatory effects by ameliorating inflammation related conditions including demyelination and neuronal degeneration (10).

In preclinical assessments for multiple sclerosis, IRAK-1 deficient mice were resistant to experimental autoimmune disease and histological analysis of their central nervous system showed no or little inflammation which indirectly down regulated T helper1 response via reduced IFN-γ production (20). IRAK1-/- T cells exhibited low expression levels of RORγt and IL-17A after treatment by TGF-β1 and IL-6 whereas Foxp3 expression enhanced which showed the critical role of IRAK1 in modulating the balance between Th17 and T regulatory cells(21). Dendritic cells of TRAF6-/- deficient mice were functionally defective in producing cytokines and up regulating co stimulatory molecules in response to CD40L along with microbial components in vitro and in vivo(22). Our data may obtain new insights into how G2013 counteract with inflammation at molecular level, their reduction by G2013 made it a new remedy for inflammation related disease and autoimmunity.

There have been disputing evidence on miR-146a expression during different types of autoimmune disease which can although affect its targets more differently (23, 24). Pauley et al showed that miR146a expression was increased in Rheumatoid Arthritis (RA) patients PBMCs while expression of IRAK1 and TRAF6 were approximately similar in RA patients and healthy controls (24). In case of lupus disease, PBMCs of patients showed high expression level of miR-146a (25, 26). Therefore reduction of NF-κB could finally reduce the inflammatory cytokines and inflammation related side effects, consequently this would lead to reduction of miR-146a in a negative indirect feedback pattern, on the other hand G2013 increased the expression of miR-146a in pre-LPS treated PBMCs in comparison with only G2013 treated group which shows that presumably G2013 competed with LPS for binding to TLR4. In fact G2013 indirectly immunomodulate induction of miR146a and NF-κB by directly targeting IRAK1 and TRAF6 in a dose dependent manner in which its high concentrations effects are more impressive than low doses thus it did not show synergistic function with miR-146a during down regulating of TLR4 signaling.

As a recommendation it would be better to evaluate the effects of G2013 on other miRNAs related to the immune system like miR-21 which aberrantly expressed in different kinds of inflammatory related disease such as cancer (27), miR-125(28), miR-126(29), miR-200(30), miR196 (31) and miR147(32) which can positively and negatively regulate IRAK1 and TRAF6. Additionally along with the miRNA network evaluating long non coding RNAs network and their possible effects on non coding RNAs network and their possible effects on miR-146a can be considered. In conclusion we can firmly express that G2013 modulates TLR4 signaling via suppression of IRAK-1 and TRAF-6 in the presence and absence of LPS but its miRNA profile is still a matter of controversy.

Acknowledgments
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References