의학석사 학위논문

사람 Telomerase 역전사효소 서열 유래 펩타이드 GV1001의 항염 효능에 대한 연구

Investigation of the anti-inflammatory effect of GV1001, a peptide derived from human telomerase reverse transcriptase (hTERT) sequence

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지도 교수 강 재 승

이 논문을 의학석사 학위논문으로 제출함

2015년 7월

서울대학교 대학원
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최 지 예

최지예의 의학석사 학위논문을 인준함

2015년 7월

위 원 장 _____ (인)
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위 원 _____ (인)

Investigation of the anti-inflammatory effect of GV1001, a peptide derived from human telomerase reverse transcriptase (hTERT) sequence

by

Jiyea Choi

A thesis submitted to the Department of medicine in partial fulfillment of the requirements for the Degree of Master of Science in Medicine (Anatomy) at Seoul National University College of Medicine

July 2015

Approved by Thesis Committee

Professor_____Chairman

Professor_____Vice chairman

Professor_____

ABSTRACT

Investigation of the anti-inflammatory effect of GV1001, a peptide derived from human telomerase reverse transcriptase (hTERT) sequence

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Department of Anatomy

Seoul National University College of Medicine

GV1001 is a peptide derived from human telomerase reverse transcriptase (hTERT) sequence, and has anti-cancer and anti-inflammatory effect. Enolase1 (ENO1) is a glycolytic enzyme and its stimulation induces to produce a large amount of pro-inflammatory cytokines from concanavalin (Con) A -activated peripheral blood mononuclear cells (PBMCs) and from ENO1 expressing monocytes and macrophages from rheumatoid

arthritis (RA) patients. However, it is still unknown whether GV1001 could regulate the ENO1-mediated pro-inflammatory cytokines production. Therefore, I investigated whether GV1001 regulates ENO1-mediated pro-inflammatory cytokines production as an anti-inflammatory peptide. First, I found that GV1001 does not affect the expression of ENO1 on Con A-activated PBMCs and RA PBMCs. However, ENO1 stimulation increased the production of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) from Con A-activated PBMCs. And it is down-regulated by the pre-treatment of GV1001. GV1001 also decreases the production of pro-inflammatory cytokines from ENO1 stimulated RA PBMCs. And then I examined what kinds of signaling molecules are involved in the down-regulation of ENO1-mediated pro-inflammatory cytokine production by GV1001. When ENO1 on the surface of Con A-activated PBMCs and RA PBMCs is stimulated, I found that p38 MAPK and NF-κB are activated. However, these are successfully suppressed by the pre-treatment of GV1001. Taken together, GV1001 might be a useful anti-inflammatory peptide via the down-regulation of pro-inflammatory cytokine production and the suppression of the

p38 MAPK and NF-κB activation by ENO1 stimulation.

Keywords: inflammation, GV1001, enolase1, rheumatoid arthritis, p38 MAPK, NF-κB

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LIST OF ABBREVIATIONS

hTERT: human telomerase reverse transcriptase

ENO1: enolase 1

RA: rheumatoid arthritis

PBMCs: peripheral blood mononuclear cells

SFMCs: synovial fluid mononuclear cells

Con A: concanavalin A

FITC: fluorescein isothiocyanate

FACS: fluorescence activated cell sorter

ELISA: enzyme-linked immunosorbent assay

IL: interleukin

CD: cluster of differentiation

TNF-α: tumor necrosis factor alpha

p38 MAPK: p38 mitogen-activated protein kinase

NF-κB: nuclear factor-kappa B

INTRODUCTION

GV1001, derived from the human telomerase reverse transcriptase (hTERT) sequence, is a peptide vaccine with 16 amino acids. It was developed as an anti-cancer agent for treatment of various cancers including advanced pancreatic cancer, non-small lung cancer and melanoma (1-5). Recent studies reported that GV1001 penetrates several cell membranes by binding with heat shock protein (HSP) and be accumulated in cytoplasm. In addition, it can be used as an effective delivery for therapeutic agents into cells. Also, this peptide protects against renal ischemia reperfusion (IRI) injury in mice by reducing acute inflammatory responses and the proportion of cell apoptosis which play key roles in causing renal injury (7). Although GV1001 has been proven to be safe in several phase Ⅰ/Ⅱ clinical trials in cancer patients (8), the mechanism of action as an anti-inflammatory agent has not been completely defined.

Enolase (ENO) is one of glycolytic enzymes, which degrades 2-phosphoglycerate to 2-phosphoenolpyruvate. It consists of three subunits depending on where it exists; α-enolase (ENO1) is expressed in most tissue, β-enolase

(ENO3) is in muscle and γ-enolase (ENO2) is in brain. ENO1 ubiquitously exists in the cytosol for its enzymatic activity in normal condition but it is expressed on cell surface in pathological condition like inflammation and cancer. Surface ENO1 acts as a plasminogen-binding receptor (9-11), which promotes plasminogen-mediated recruitment of monocytes to induce acute inflammation in lung and pneumonia patients exhibit elevated levels of ENO1 on the surface of PBMCs and intense ENO1 staining on mononuclear cells in the alveolar compartment (12). Many studies have reported that ENO1 expression is increased on cell surface by various stimulations and it is involved in many functions for inflammatory responses. Lipopolysaccharide (LPS) stimulation increased the translocation of ENO1 from cytosol to cell surface in primary monocyte and human monocytic cell line, U937 (12). The stimulation of ENO1 on hematopoietic cells, such as neutrophils, lymphocytes, and monocytes, by phorbol myristate acetate (PMA) increased capacity to generate plasmin (13, 14). In our previous study, Con A stimulation increased the expression of ENO1 on the surface of PBMCs and consequently increased the production of pro-inflammatory cytokines (15). It

is widely known that inflammatory response in Con A-activated PBMCs by ENO1 stimulation is similar with the response in PBMCs from rheumatoid arthritis (RA) patients. There are many reports that ENO1 antibodies play pathogenic roles in a variety of autoimmune and inflammatory diseases such as systemic lupus erythematous, systemic sclerosis, Behcet's disease, ulcerative colitis, Crohn's disease, retinopathy and RA (16-18). RA is a representative autoimmune disease including synovial inflammation, pannus formation and subsequent bone destruction (19-25). Many cell types such as monocytes and macrophages were infiltrated in inflamed sub-synovium, which plays key patho-physiological roles in RA (26-28). Furthermore, these cells lead to continuous inflammation and exacerbate symptoms by up-regulating the production of PGE_2 and several cytokines such as TNF-α, IL-1, IL-6, and IFN-γ (15, 29, 30). It means that ENO1 exacerbates pathogenicity of RA (31). Its expression on the surface of monocytes and macrophages of RA is more increased than that of healthy control. The higher ENO1 expressed on RA PBMCs is correlated with the abundant production of pro-inflammatory cytokines through p38 MAPK

and NF-κB pathway (15).

Therefore, I investigated whether GV1001 has an anti-inflammatory effect on ENO1-induced inflammatory response via down-regulation of pro-inflammatory cytokine production and its related mechanisms.

MATERIALS AND METHODS

Isolation of PBMCs Heparinized peripheral blood was obtained from healthy volunteers and RA patients, after approved by institutional review board of Seoul National University Hospital. Blood was mixed with equal volume of phosphate buffered saline (PBS). And then PBMCs were isolated with density gradient centrifuge by using Ficoll-Paque^{TM} PLUS (Amersham Pharmacia Biotech, Piscataway, NJ, USA). After centrifugation, buffy coat was collected and washed twice with PBS. Red blood cell (RBC) was lysed with RBC lysis buffer (Sigma, St. Louis, MO. USA) by shaking for 5 min at 37℃. After cells were washed with PBS twice, cell number was counted and its purity was examined by flow cytometry.

Stimulation of PBMCs with Con A To induce ENO1 expression on the surface of PBMCs, isolated PBMCs were stimulated with Con A (2 μg/ml) and then cultured in complete RPMI1640

(Welgene, Korea) media containing 10% heat inactivated fatal bovine serum (GE Health care Hyclone, UT, USA) and antibiotics (100 U/ml of penicillin and 100 ㎍/ml streptomycin; Welgene, Korea) for 24 hrs. After Con A stimulation, PBMCs were washed with PBS twice and re-suspended into completed RPMI1640.

Prepration of Anti-ENO1 monoclonal antibody Anti-ENO1 mAb was separated and purified from the ascites of mice infected with F6 (hybridoma producing anti-ENO1 mAb) using a protein A column (Pierce, Grand Island, NY, USA). Ascites was diluted to 1:1 ratio with Protein A IgG Binding buffer (Pierce, Grand Island, NY, USA) and centrifuged at 10,000g for 20 min. After filtration with 0.22 ㎛ syringe filter, diluted ascites applied to the column and allowed to flow completely into the resin bed. After flowing the diluted ascites, column was flowed with 15 ml of Binding buffer and 5 ml of Elution buffer (Pierce, Grand Island, NY, USA) to elute antibodies. The concentration of separated antibodies was measured by BCA assay. For flow cytometry analysis, anti-ENO1 mAb was labeled with Fluorescein

isothiocyanate (FITC) by FluroTag™ FITC conjugation kit (Sigma, St. Louis, MO. USA). And then FITC-conjugated anti-ENO1 mAb was diluted with PBS (1 mg/ml) and stored at 4℃.

ENO1 stimulation PBMCs ($1x10^6$/ml) from healthy control and RA patients were stimulated with anti-ENO1 mAb (1 mg/ml) for 1 hr at room temperature with gentle rotation. MOPC21 (1 mg/ml: Sigma) was used as an isotype control. After ENO1 stimulation, cells were seeded on 24-well plates and incubated at 37℃ in a humidified incubator with 5% CO_2.

GV1001 treatment GV1001 was provided by KAEL-GemVax (Seongnam, Korea). GV1001 was dissolved in PBS. The final concentration was 10 mM. After Con A-activated PBMCs and RA PBMCs ($4x10^6$/ml) were pre-treated with GV1001 (100 μ M) for 1 hr at room temperature with gentle rotation, cells were stimulated with anti-ENO1 mAb as described above.

Flow cytometry analysis PBMCs ($1x10^5$cells/each group) were re-suspended in PBS containing 0.5% BSA and 0.1% sodium

azide (FACS buffer). After PBMCs were stained with FITC-conjugated anti-ENO1 mAb or isotype control (BD Bioscience, San Jose, CA, USA) for 30 min at 4℃. Cells were also stained with the PE-conjugated anti-human CD69 (BD Biosciences), CD25 (BD Biosciences), CD3 (BD Biosciences), CD14 (BD Biosciences) antibodies or its isotype control (Sigma). After washing with FACS buffer, the surface expression of ENO1 was analyzed by a FACS Calibur flow cytometer (BD Biosciences) and FlowJo software (BD Biosciences). At least 10,000 cells were analyzed per condition.

Enzyme-Linked Immunosorbent Assay (ELISA) After GV1001 pre-treatment and ENO1 stimulation, PBMCs were incubated for 48 hrs for Con A-activated PBMCs or 12 hrs for RA PBMCs and culture supernatant was collected. The levels of TNF-α, IL-1β (Biolegend, CA, USA) and IL-6 (R&D systems, CA, USA) in the supernatant were measured using ELISA kits according to the manufacturer's instructions. The relative absorbance was measured at 450 nm using the SoftmaxPro software (Molecular Devices, sunnyvale, CA, USA).

Inhibitor study for signal pathway To identify the GV1001-related mechanism, p38 MAPK (SB203580) and NF-κB (Bay11-7082) inhibitors were purchased from Sigma. SB203580 (40 μM), Bay11-7082 (2.5 μM), GV1001 (100 μM) and DMSO (vehicle control) were pre-treated for 1 hr and then PBMCs were stimulated with anti-ENO1 mAb for 1 hr. These cells were incubated for further 48 hrs and then supernatant was collected for ELISA.

Immunoblotting Cells ($1x10^6$) were lysed with lysis buffer containing 50 mM Tris-HCl (pH 7.4), 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, protease inhibitor cocktails and phosphatase inhibitor cocktails (Sigma). The protein concentration was measured with BCA assay. An equal amount of protein (10 μg/sample) was separated by 10% SDS-PAGE with 50-80V for 4 hrs, and transferred onto a nitrocellulose membrane with 400 mA for 1 hr. After blocking with 5% nonfat milk in PBS containing 0.05% Tween 20 (PBST) for 1 hr at room temperature, the membrane was incubated with primary Ab at 4℃ overnight. Primary Abs for phospho-p38 MAPK (1:500;

Cell signaling Technology, Danvera, MA, USA), p38 MAPK (1:1000; Santa Cruz Biotechnology, TX, USA), phospho-p65 (1:500; Santa Cruz Biotechnology), p65 (1:1000; Santa Cruz Biotechnology) and β-actin (1:5000; Sigma) were diluted with 1% nonfat milk in PBST. After washing 3 times (5 min/each) with PBST, membrane was incubated with horse radish peroxidase (HRP)-conjugated anti-rabbit IgG (1:5000; Cell signaling Technology) for phospho-p38 MAPK, p38 MAPK, phospho-p65 NF-κB, p65 NF-κB and mouse HRP-conjugated anti-mouse IgG (1:5000; Cell signaling Technology) for β-actin for 1 hr at RT. The membrane was then washed 3 times (5 min/each) with PBST and the EZ-Western Lumi La (Dogen, Daeill, Korea) solution was loaded to detect the signal. The density of bands was analyzed using Image J software (NIH, Bethesda, MD, USA). Results were expressed as relatve intensity and each band was adjusted to that of p38 MAPK or p65 NF-κB.

Statistical analysis Data are presented as mean ± SD. The Newman-keuls ANOVA was use to compared groups. Statistical

values were carried out using GraphPad InStat version 5.01 (GraphPad Software, La Jolla, CA, USA).

RESULTS

1. Con A stimulation increases ENO1 expression on the surface of PBMCs

It is generally known that ENO1 is not or lowly expressed on the surface of inactivated PBMCs from healthy volunteer, but it is considerably increased by Con A stimulation (15). When PBMCs were treated with Con A for 24 hrs, they showed increased size and granularity (Fig. 1A). And expression of activation markers, CD69 and CD25, was increased (Fig. 1B and D). In addition, ENO1 expression on PBMCs was increased by Con A stimulation (Fig. 2A). To clarify which cells express ENO1, Con A-stimulated PBMCs were stained with antibody against ENO1, CD3, a marker of T cells, and CD14, a marker of monocytes and macrophages. As a result, ENO1 was mainly expressed on $CD14^+$ cells in Con A-activated PBMCs (Fig. 2B and C).

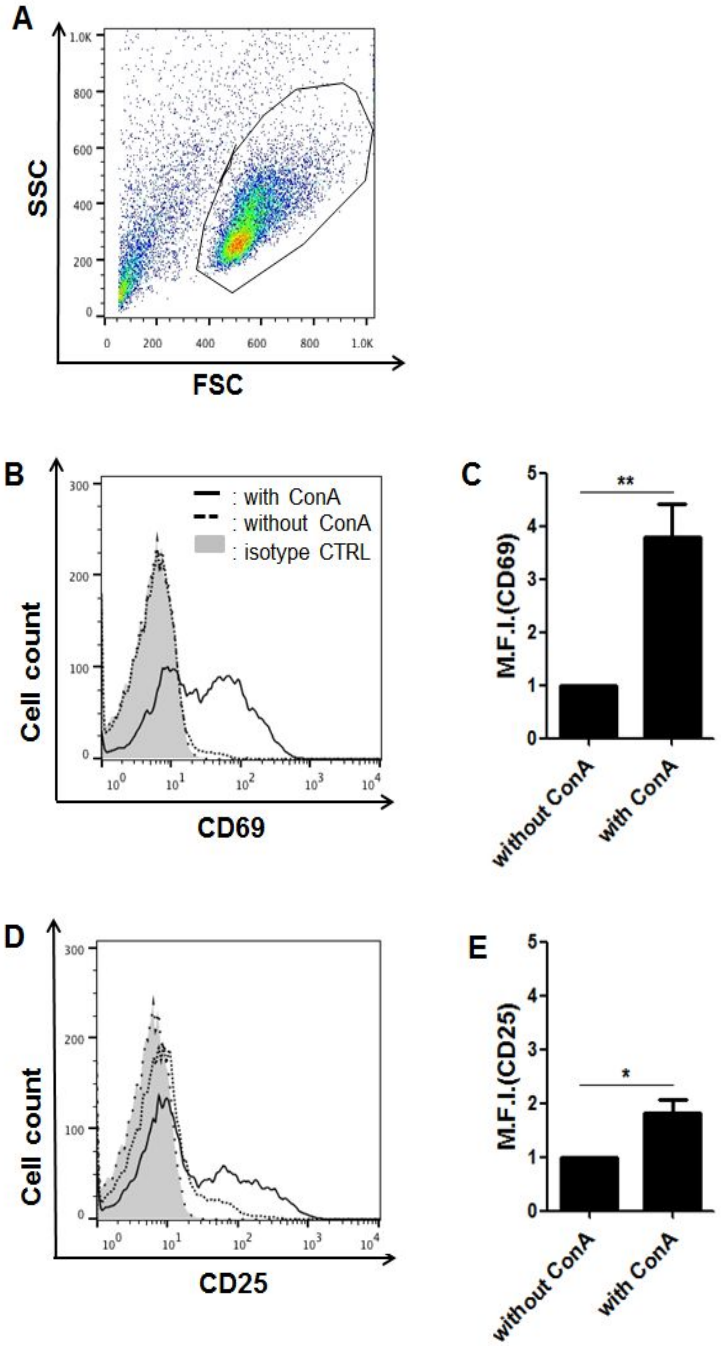

A
SSC
FSC
B
: with ConA
: without ConA
: isotype CTRL
Cell count
CD69
C
M.F.I.(CD69)
**
without ConA
with ConA
D
Cell count
CD25
E
M.F.I.(CD25)
*
without ConA
with ConA

Fig. 1 Activation of PBMCs by Con A stimulation.

PBMCs obtained from healthy control ($1x10^6$/ml) were stimulated with Con A (2 μg/ml) for 24 hrs. (A) Size and granularity of Con A-activated PBMCs were increased. After washing, cells ($1x10^5$/group) were stained with (B) PE-conjugated anti-CD69 Ab or (D) PE-conjugated anti-CD25 Ab. (C) CD69 expression was represented with Mean Fluorescence Intensity (MFI) of cells stained with anti-CD69 Ab (n=5). (E) CD25 expression was represented with MFI of cells stained with anti-CD25 Ab (n=5). PE-conjugated MOPC21 were used as an isotype control.

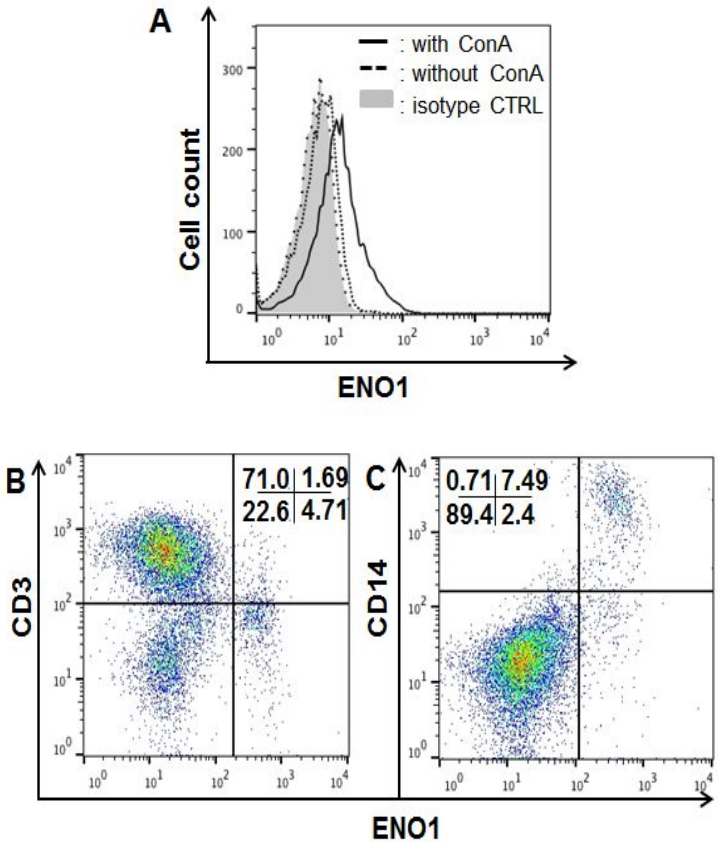


Fig. 2 Increased ENO1 expression on PBMCs by Con A stimulation.

Con A-stimulated PBMCs were stained with (A) FITC-conjugated anti-ENO1 mAb alone or combined with (B) PE-conjugated anti-CD3 Ab and (C) PE-conjugated anti-CD14 Ab. FITC- or PE- conjugated MOPC21 were used as isotype control.

2. GV1001 doesn't affect ENO1 expression on PBMCs by Con A treatment

The effect of GV1001 on the production of pro-inflammatory cytokines by ENO1 stimulation was examined. First, I investigated whether GV1001 could change Con A-induced ENO1 expression on the surface of PBMCs. After cells were treated with GV1001 before or after Con A stimulation, the changes of the expression of ENO1 on surface were analyzed by flow cytometry. As a result, there was no remarkable change on ENO1 expression between pre- and post-treatment of GV1001 (Fig. 3A and B). Therefore, GV1001 doesn't affect Con A-induced ENO1 expression on the surface of PBMCs.

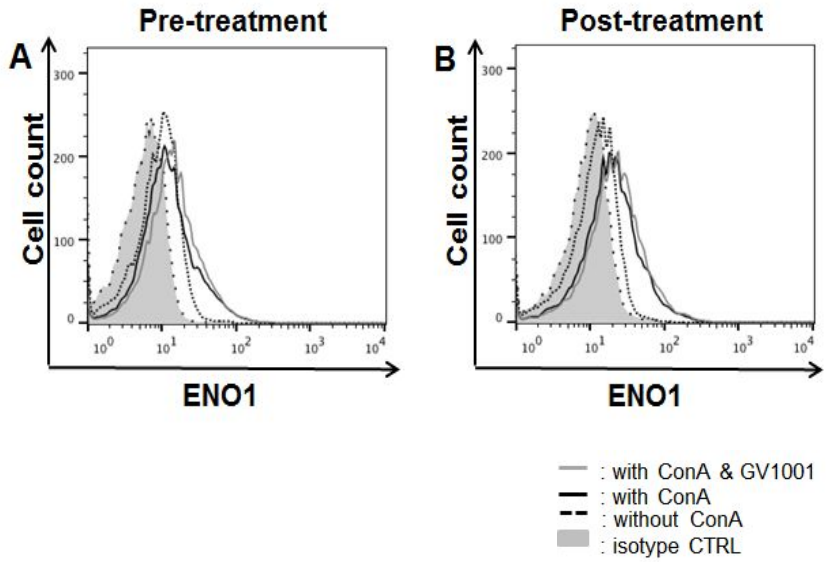


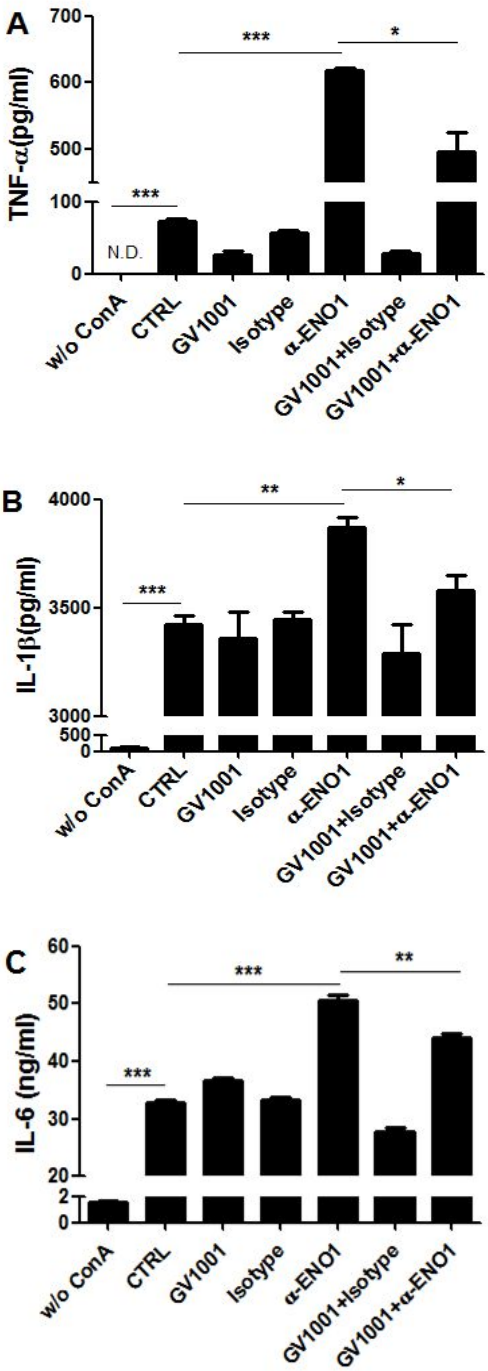
Fig. 3 The effect of GV1001 on ENO1 expression.

(A) PBMCs ($1x10^6$/ml) were pre-treated with GV1001 (100 μM) or vehicle for 1 hr and then cultured in presence of Con A (2 μg/ml) or absence for 24 hrs. Then, cells of each group were harvested, washed with PBS and further incubated for 48 hrs. (B) PBMCs ($1x10^6$/ml) were stimulated with Con A (2 μg/ml) or vehicle for 24 hrs. And, cells were washed with PBS and post-treated with GV1001 (100 μM) for 1 hr and then incubated for further 48 hrs. ENO1 expression of these groups were analyzed by flow cytometry.

3. GV1001 decreases the production of TNF-α, IL-1β and IL-6 by ENO1 stimulation

It is known that ENO1 stimulation induces inflammation through increasing the production of pro-inflammatory cytokines (15). There is a recent report that administration of GV1001 reduced the infiltration of macrophages in renal ischemia reperfusion injury in mice, and it suppressed the production of IL-6 and MCP-1 (7). Thus, I examined whether the level of pro-inflammatory cytokines was increased by ENO1 stimulation with anti-ENO1 mAb, and then whether GV1001 could suppress the production of pro-inflammatory cytokines from Con A-activated PBMCs by ENO1 stimulation. PBMCs were pre-treated with GV1001 for 1 hr and then cells were simulated with anti-ENO1 mAb for another 1 hr. After cells were incubated for further 48 hrs, the levels of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 were measured by ELISA. When ENO1 expressing PBMCs were stimulated with anti-ENO1 mAb, the levels of TNF-α (anti-ENO1 Ab (617.6.1±7.7 pg/ml) vs. control (72.4±9.07 pg/ml), $p<0.001$), IL-1β (anti-ENO1 Ab (3872.2±75.7 pg/ml) vs. control

(3427.0±66.2 pg/ml), $p<0.01$) and IL-6 (anti-ENO1 Ab (50.66±1.39 ng/ml) vs. control (32.77±0.72 ng/ml), $p<0.001$) were dramatically increased (Fig. 4). But, they were considerably reduced (TNF-α: GV1001+anti-ENO1 Ab (496.2±51.9 pg/ml) vs. anti-ENO1 Ab (617.6±7.7 pg/ml), $p<0.05$; IL-1β: GV1001+anti-ENO1 Ab (3578.0±122.0 pg/ml) vs. anti-ENO1 Ab (3872.2 ± 75.7 pg/ml), $p<0.05$ and IL-6: GV1001+anti-ENO1 Ab (43.96 ± 1.25 ng/ml) vs. anti-ENO1 Ab (50.66± 1.39 ng/ml), $p<0.01$) by GV1001 pre-treatment (Fig. 4). These results suggest that GV1001 suppresses the production of pro-inflammatory cytokines, which are produced from Con A-activated PBMCs by ENO1 stimulation.


A
TNF-α(pg/ml)
700
600
500
100
0
N.D.
***
***
*
w/o ConA
CTRL
GV1001
Isotype
α-ENO1
GV1001+Isotype
GV1001+α-ENO1
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IL-1β(pg/ml)
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3500
3000
500
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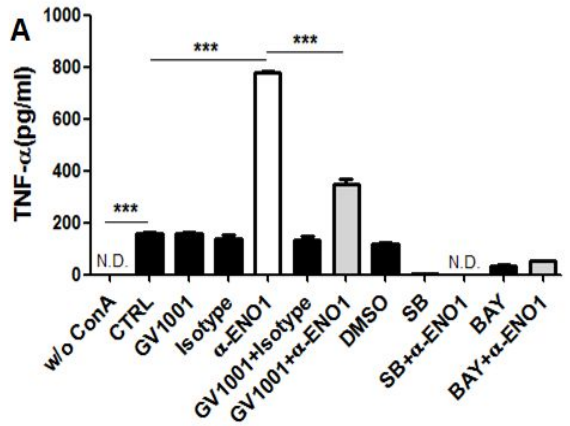
*
***
C
IL-6 (ng/ml)
60
50
40
30
20
2
0
***
**
***

Fig. 4 The inhibitory effect of GV1001 on ENO1-induced pro-inflammatory cytokines production from Con A-activated PBMCs.

Isolated PBMCs ($1x10^6$/ml) from normal individuals were stimulated with Con A (2 μg/ml) for 24 hrs. Activated PBMCs were pre-treated with GV1001 (100 μM) for 1 hr and stimulated with anti-ENO1 mAb (1 μg) for 1 hr. MOPC21 was used as an isotype control. Stimulated PBMCs were cultured in 24-well plate ($1x10^6$/ml) for further 48 hrs. The culture supernatant of stimulated PBMCs was collected and the production of (A) TNF-α, (B) IL-1β and (C) IL-6 was measured by ELISA. Each sample is in triplicates and results are representative of three independent experiments. Data are presented as the means ± SD. *p<0.05, **p<0.01, ***p<0.001.

4. GV1001 suppresses the activation of p38 MAPK and NF-κB in Con-A activated PMBCs: ELISA

As shown in Fig. 4, GV1001 suppressed the ENO1-induced production of TNF-α, IL-1β and IL-6. I next examined its related signaling mechanism. Recently, ENO1 was reported to enhance the production of pro-inflammatory mediators via the activation of p38 MAPK and NF-κB (15). It suggests that GV1001 might interrupt p38 MAPK and NF-κB signaling pathway. Therefore, Con A-activated PBMCs were pre-treated with p38 MAPK inhibitor (SB203580), NK-κB inhibitor (Bay11-7082) and GV1001 to for 1 hr and cells were stimulated with anti-ENO1 mAb then cultured for further 48 hrs. TNF-α, IL-1β and IL-6 in the culture supernatants were measured by ELISA. Like the suppression of TNF-α, IL-1β and IL-6 production by SB203580 and Bay11-7082, pre-treatment of GV1001 also suppressed the ENO1-induced TNF-α, IL-1β and IL-6 production (Fig. 5). Thus, it seems that the pre-treatment of GV1001 suppresses the ENO1-induced TNF-α, IL-1β and IL-6 production through the inhibition of the activation p38 MAPK and NF-κB.


A
TNF-α(pg/ml)
1000
800
600
400
200
0
***
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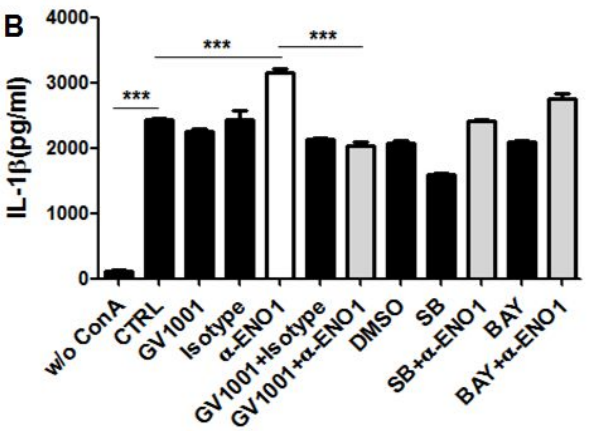
***
N.D.
N.D.
w/o ConA
CTRL
GV1001
Isotype
α-ENO1
GV1001+Isotype
GV1001+α-ENO1
DMSO
SB
SB+α-ENO1
BAY
BAY+α-ENO1


B
IL-1β(pg/ml)
4000
3000
2000
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***
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w/o ConA
CTRL
GV1001
Isotype
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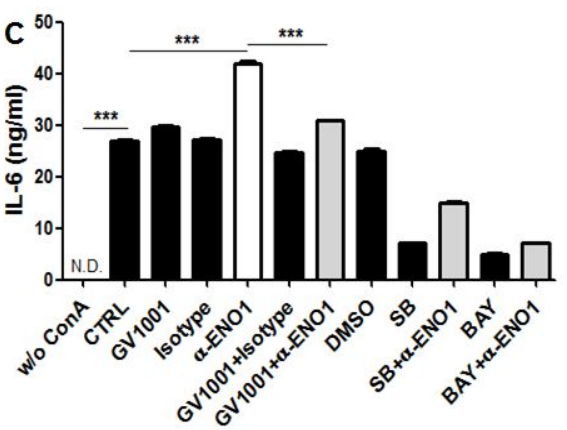
GV1001+Isotype
GV1001+α-ENO1
DMSO
SB
SB+α-ENO1
BAY
BAY+α-ENO1


C
IL-6 (ng/ml)
50
40
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0
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N.D.
w/o ConA
CTRL
GV1001
Isotype
α-ENO1
GV1001+Isotype
GV1001+α-ENO1
DMSO
SB
SB+α-ENO1
BAY
BAY+α-ENO1

Fig. 5 The regulation of p38 MAPK and NF-κB activation by GV1001 treatment

Con A-activated PBMCs were pre-treated with GV1001 (100 μM), DMSO (vehicle), SB203580 (40 μM) and Bay11-7082 (2.5 μM) for 1 hr and then stimulated with anti-ENO1 mAb for 1 hr. After incubating for another 48 hrs, the supernatant was harvested and pro-inflammatory cytokine's levels were determined by ELISA. (A) TNF-α, (B) IL-1β and (C) IL-6. Data are presented as the means ± SD. ***p<0.001.

5. GV1001 suppresses the activation of p38 MAPK and NF-κB in Con-A activated PMBCs: Immnunoblotting

To confirm the inhibition of the activation of p38 MAPK and NF-κB by GV1001, immunoblotting was performed. I first did time kinetic study to find the optimal time for the activation of p38MAPK and NF-κB by ENO1 stimulation. Con A-activated PBMCs were stimulated with anti-ENO1 mAb for 30, 60, 90 and 120 min, and then p38 MAPK activation was examined by immunoblotting. In case of NF-κB activation, immunoblotting was performed after stimulation with anti-ENO1 mAb for 5, 10, 15, and 30 min. As a result, at 60 min after stimulation, the activation of p38 MAPK increases and its peak was at 120 min after ENO1 stimulation (Fig. 6A and B). It is generally known that the activation of NF-κB is relatively earlier than the activation of p38 MAPK. NF-κB activation was peaked at 5 min after ENO1 stimulation and quickly diminished (Fig. 7A and B). Based on these results, I verified whether GV1001 could interrupt ENO1-induced activation of p38 MAPK and NF-κB . As shown in Fig. 8 and 9, phosphorylated p38 and p65 were increased by ENO1 stimulation, but it was efficiently suppressed

by the pre-treatment of GV1001. Taken together, GV1001 shows anti-inflammatory effect via the inhibition of the activation of p38 MAPK and NF-κB.

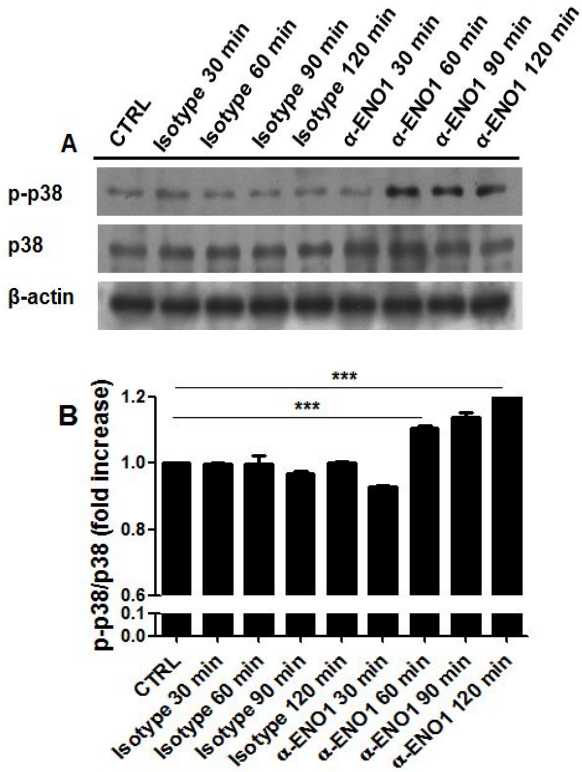

Fig. 6 Time kinetic study for p38 MAPK activation by ENO1 stimulation.

PBMCs ($1x10^6$/ml) were incubated with Con A (2 μg/ml) for 24 hrs and stimulated with anti-ENO1 mAb for 30, 60, 90 and 120 min. The expression of (A) phophorylated p38 (p-p38) and total p38 was examined by immunoblotting. The relative fold increase of (B) p-p38 to p38 was represented after densitometry analysis. Results are representative of three independent experiments. ***$p<0.001$

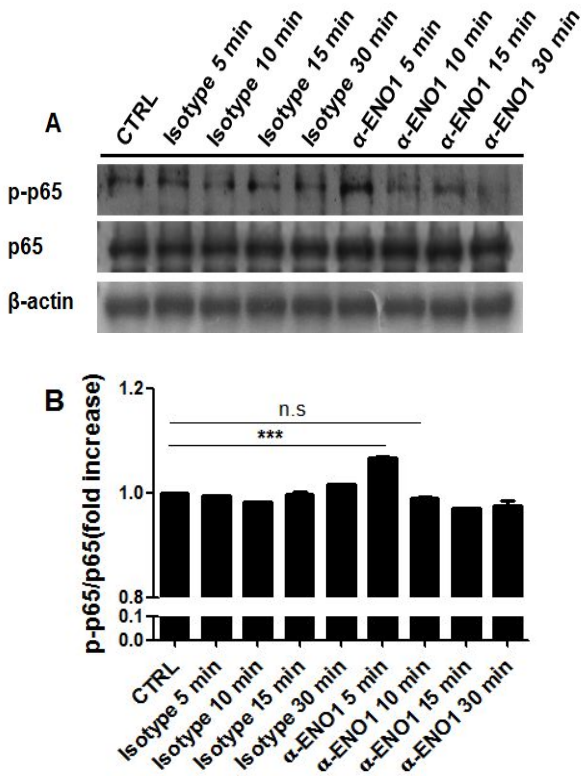

Fig. 7 Time kinetic study for NF-κB activation by ENO1 stimulation.

PBMCs ($1x10^6$/ml) were incubated with Con A (2 μg/ml) for 24 hrs, and stimulated with anti-ENO1 mAb for 5, 10, 15 and 30 min. The expression of (A) phophorylated p65 (p-p65) and total p65 was examined by immunoblotting. The relative fold increase of (B) p-p65 to p65 was represented after densitometry analysis. Results are representative of three independent experiments. ***p<0.001

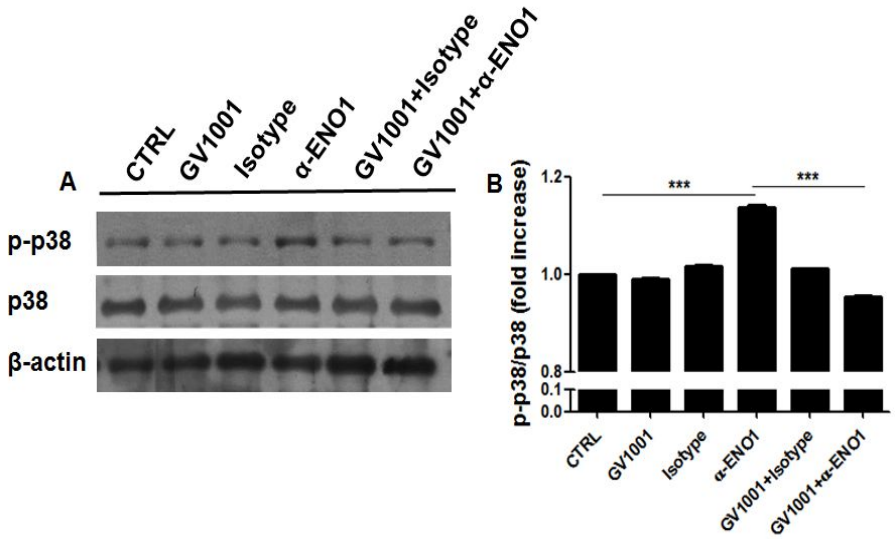

Fig. 8 The suppression of p38 MAPK activation by GV1001 treatment.

Con A-activated PBMCs were pre-treated with GV1001 (100 μM) for 1 hr, and stimulated with anti-ENO1 mAb for 120 min. Then, cells were collected and lysed with lysis buffer to extract protein for immunoblotting. Levels of (A) phophorylated form of p38 (p-p38) and total p38 was examined by immunblotting. Densitometry analysis was performed and it was represented as fold increase of phosphorylated form to total form, (B) p-p38/p38. It was represented as three independent experiments. **p<0.01, ***p<0.001.

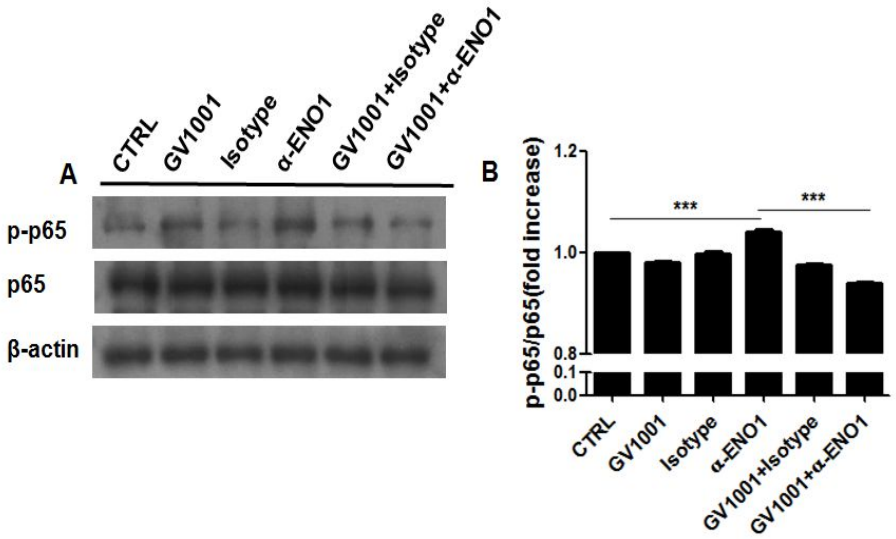

Fig. 9 The suppression of NF-κB activation by GV1001 treatment.

Con A-activated PBMCs were pre-treated with GV1001 (100 μM) for 1 hr, and stimulated with anti-ENO1 mAb for 5 min. Then, cells were collected and lysed with lysis buffer to extract protein for immunoblotting. Levels of (A) phophorylated form of p65 (p-p65) and total p65 was examined by immunblotting. Densitometry analysis was performed and represented as fold increase of phosphorylated form to total form, (B) p-p65/p65. It was represented as three independent experiments. **p<0.01, ***p<0.001.

6. GV1001 doesn't change the expression level of ENO1 on PBMC from RA patients

It is recognized that inflammatory responses in PBMCs induced by Con A stimulation are similar with the inflammatory responses in rheumatoid arthritis disease which is a systemic inflammatory, autoimmune disease and characterized by severe inflammation at joint and destruction of bone and cartilage (27, 28). It is previously reported that the level of ENO1 expression on PBMCs from RA patients was much higher than that on PBMCs from normal healthy individuals. Monocytes and macrophages, which causes severe acute inflammation, are major ENO1-positive immune cells (15). Therefore, the anti-inflammatory effect of GV1001 in RA PBMCs was also confirmed as in Con A-stimulated PBMCs. Most PBMCs were spontaneously activated in the RA patients (Fig. 10A). And ENO1 was also up-regulated in RA PBMCs (Fig. 10B). Like in the report that ENO1 is mostly expressed on monocytes and macrophages from RA patients (15), I confirmed most $CD14^+$ cells were expressed ENO1 on their surface. Since GV1001 doesn't affect ENO1 expression on Con A-activated PBMCs, I

examined whether GV1001 changes ENO1 expression on the surface of PBMCs from RA patients. As a result, ENO1 expression on $CD14^+$ cells did not be changed by GV1001 treatment (Fig. 10C and D). It suggests that treatment of GV1001 does not change ENO1 expression on PBMCs from RA patients.

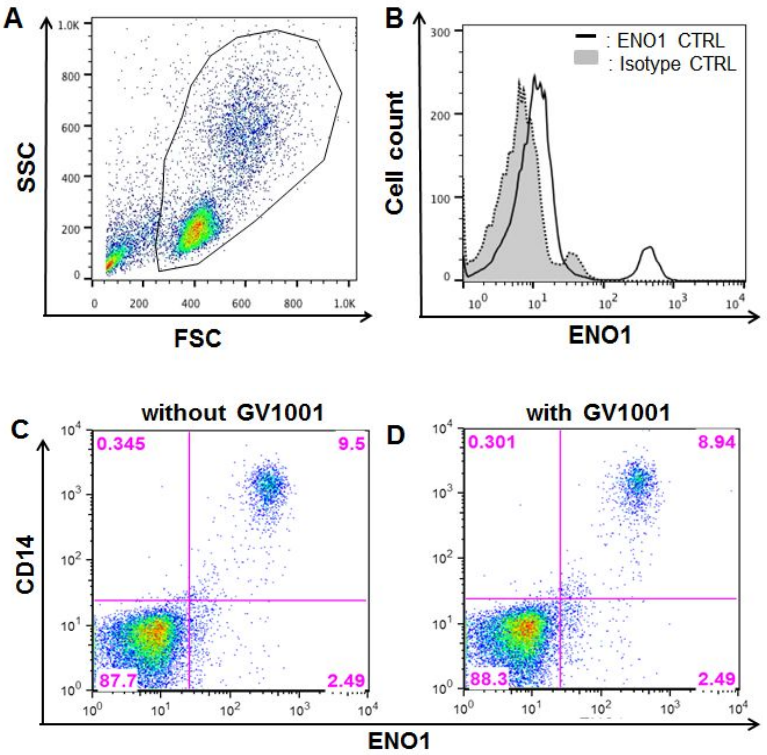

Fig. 10 The effect of GV1001 on ENO1 expression in RA PBMCs.

PBMCs ($1x10^5$) separated from RA patients were stained with anti-ENO1 mAb or isotype control. (A) Size and granularity and (B) ENO1 expression was examined by flow cytometry. RA PBMCs were incubated with the (C) absence or (D) presence of GV1001 (100 μM) for 48 hrs. After the washing, cells were double-stained with FITC conjugated anti-ENO1 mAb and PE-conjugated anti-CD14 Ab.

7. GV1001 decreases the production of TNF-α, IL-1β and IL-6 from RA PBMCs by ENO1 stimulation

GV1001 suppressed the induction of pro-inflammatory cytokines from Con A-activated PBMCs (Fig. 4). Therefore, the anti-inflammatory effect of GV1001 was also verified in RA PBMCs. After RA PBMCs were incubated with GV1001 for 1 hr before ENO1 stimulation, cytokine levels were measured by ELISA. The amount of TNF-α (anti-ENO1 Ab (719.3 ± 12.6 pg/ml) vs. control (34.9 ± 21.9 pg/ml), $p<0.001$), IL-1β (anti-ENO1 Ab (321.5 ± 17.4 pg/ml) vs. control (36.9 ± 2.7 pg/ml), $p<0.001$) and IL-6 (anti-ENO1 Ab (2.45 ± 1.31 ng/ml) vs. control (1.63 ± 2.54 ng/ml), $p<0.001$) were surged by ENO1 stimulation, and it was diminished by pre-treatment of GV1001 (TNF-α; GV1001+anti-ENO1 Ab (341.0 ± 8.1 pg/ml) vs. anti-ENO1 Ab (719.3 ± 12.6 pg/ml), $p<0.001$, IL-1β; GV1001+anti-ENO1 Ab (224.3 ± 7.6 pg/ml) vs. anti-ENO1 Ab (321.5 ± 17.4 pg/ml), $p<0.001$ and IL-6; GV1001+anti-ENO1 Ab (1.99 ± 1.02 ng/ml) vs. anti-ENO1 Ab (2.45 ± 1.31 ng/ml), $p<0.01$). It suggests that GV1001 may alleviate robust inflammation by ENO1 stimulation in RA PBMCs.

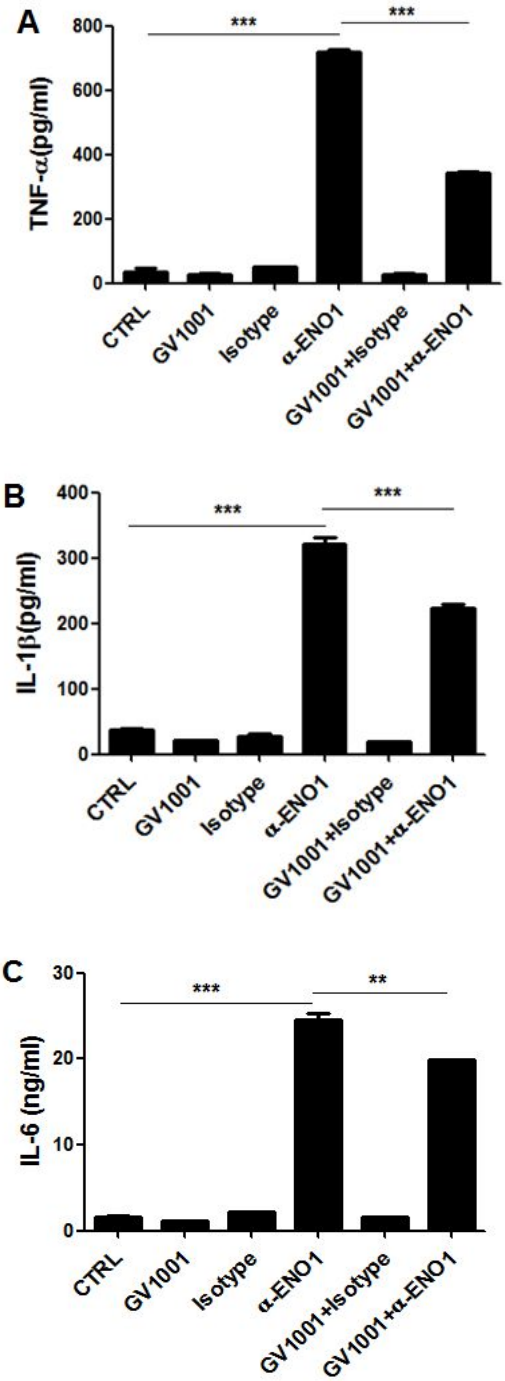
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Fig. 11 The effect of GV1001 on ENO1-induced pro-inflammatory cytokines production from RA PBMCs.

RA PBMCs were pre-treated with GV1001 (100 μM) for 1 hr and then incubated with anti-ENO1 mAb (1 μg) or MOPC21 isotype control for 1 hr. After stimulated cells were seeded in 24-well plate, they were further incubated for 12 hrs. The culture supernatant were collected and the levels of (A) TNF-α, (B) IL-1β and (C) IL-6 were measured by ELISA. Each sample is in triplicates and results are representative of three independent experiments. Data are presented as the means ± SD. **p<0.01, ***p<0.001.

8. GV1001 suppresses the activation of p38 MAPK and NF-κB in RA PMBCs.

GV1001 suppressed the activation of p38 MAPK and NF-κB in Con A-activated PBMCs by ENO1 stimulation (Fig. 8 and 9). Next, it was examined whether it could suppress the activation of p38 MAPK and NF-κB in PBMCs from RA patients. After RA PBMCs were pre-treated with GV1001 for 1 hr and stimulated by anti-ENO1 mAb for another 1 hr. Based on time kinetic study shown in Fig. 6, the phosphoryolated p38 and p65 was determined at 120 min after ENO1 stimulation by immunoblotting (Fig. 12). As a result, the phosphorylation of p38 and p65 was increased by ENO1 stimulation, which was effectively reduced by pre-treatment of GV1001.

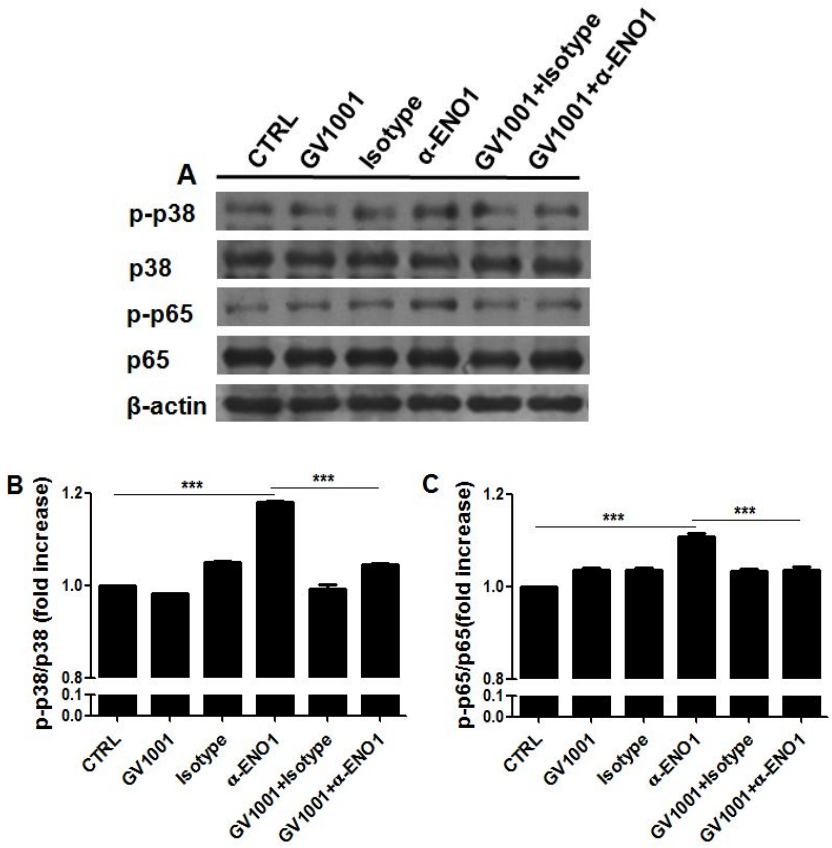

Fig. 12 The suppression of p38 MAPK and NF-κB activation in RA PBMCs by GV1001-treatment.

RA PBMCs were stimulated with anti-ENO1 mAb for 120 min after pre-treatment of GV1001 (100 μM) for 1hr. (A) The phosphorylation of p38 and p65 was detected by immunoblotting. The relative expression was represented as fold increase of (B) p-p38/p38 or (C) p-p65/p65 by densitometry analysis. Results are representative of three independent experiments. ***p<0.001.

DISCUSSION

RA is an autoimmune disease which affect up to 3% of the population (32). In the past few years, many researchers have tried to elucidate the causes of RA and to develop therapeutic approaches. There are many causes that get a RA and make it worse, and one of them is the network of various cytokine such as TNF-α, IL-1 and IL-6 produced by infiltrated cells such as monocytes and macrophages in inflamed synovial joint (33-35). However, the precise reasons and therapies for RA have not been completely figured out. The role of ENO1 on the pathogenesis RA and its possible role as a therapeutic agents are reported by my colleagues (15).

ENO1, an essential enzyme involving in glycolysis, is located in the cytoplasm in a normal condition, but translocated to the cell membrane in a pathogenic status. Recent studies demonstrated that ENO1 is a new putative target for auto-antigen in RA (16). Also, an increase of ENO1-positive cells induces a swift migration of inflammatory cells into the lung inflammatory lesion (23). It is previously reported that ENO1 is over-expressed on monocytes and macrophages in PBMCs and

SFMCs of RA patients (15). When the translocated ENO1 from cytosol to cell surface is stimulated, the production of PGE_2 and pro-inflammatory cytokines such as TNF-α, IL-1α/β, IFN-γ, and IL-18 are increased. All of them are closely related to pathogenesis of RA, and also promote inflammatory cell recruitment to the inflamed synovial tissue. Therefore, the investigation regarding the molecules that regulate production of pro-inflammatory cytokines will provide a clue for RA treatment.

GV1001 is developed for a cancer vaccine (1-3) and also has a function as an anti-inflammatory agent (7). In renal IRI mouse model, this peptide efficiently inhibited the production of IL-6 and MCP-1, which were associated with the decrease in infiltration of neutrophils and macrophages in the kidney after IRI. Futhermore, GV1001 interacts with various proteins for its action mechanism in the cytoplasm, and ENO1 is one of these proteins (6). Hence, it seemed that GV1001 could be used as an effective therapeutic agent for RA via the regulation of ENO1 activity. In fact, I showed the effect of GV1001 on the regulation of RA pathogenesis via the down-regulation of TNF-α, IL-1β, IL-6 production. It means that GV1001 is not only the effective

therapeutic agents, but also acts as a adjuvant for the conventional RA therapeutic drugs.

Several pro-inflammatory cytokines directly affect the pathogenicity of RA. In case of TNF-α, a NF-κB activation cascade initiator, induces the production of other pro-inflammatory cytokines through NF-κB signal pathway (36). NF-κB signal forms a positive regulatory cycle that may amplify and maintain the process of RA disease (36). IL-6 activates the Janus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT) pathway, and STAT-3 is activated in RA constitutively (37, 38). The inflammatory process is usually tightly regulated by mediators that initiate and maintain inflammation and that shut the process down. Effort to develop more effective treatments for RA is based on understanding of cytokine network. Over-production of pro-inflammatory cytokines has been targeted with therapeutic antibodies or receptor antagonists. Against this pathogenicity of RA, many therapeutic agents such as Tocilizumab and Infiximab developed. These are immunosuppressive drugs, and each attenuates the IL-6 and TNF-α-mediated signaling by blocking the

combination of cytokines and their receptors. Although these are effective inflammatory inhibitors, there are several adverse side effects such as upper respiratory tract infection and serious infection to other multiple organs (39-42). In the current study, Con A-activated PBMCs and RA PBMCs produced high levels of pro-inflammatory cytokines by ENO1 stimulation. However, treatment of GV1001 before ENO1 stimulation decreased the production of these cytokines that are directly related with inflammation (Fig. 4 and 11). Therefore, GV1001 is a capable suppressor and candidate for therapeutic agents via reducing ENO1-mediated inflammation.

It is well known that ENO1 induces inflammation via the activation of p38 MAPK and NF-κB signal pathway in RA (15). These signal pathways play important roles in pathogenesis of RA. NF-κB acts as a pivotal regulator of inflammation via the transcriptional regulation. It is closely involved in the development of T helper 1 (Th1) responses, the activation of abnormal apoptosis and the proliferation of RA fibroblast-like synovial cells (43, 44). Also, p38 MAPK pathway in RA contributes to the up-regulation of pro-inflammatory cytokines,

chemokines, and metalloproteinases (45). As shown in Fig. 8, 9 and 12, ENO1 stimulation significantrly activates p38 MAPK and NF-κB signal pathway, but it is effectively inhibited by pre-treatment of GV1001 before ENO1 stimulation.

Taken together, GV1001 might be useful anti-inflammatory peptide via the down-regulation of pro-inflammatory cytokine production and the suppression of the p38 MAPK and NF-κB activation by ENO1 stimulation.

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국 문 초 록

사람 Telomerase 역전사효소 서열 유래 펩타이드 GV1001의 항염 효능에 대한 연구

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사람 telomerase 역전사 효소 서열로부터 유래된 GV1001은 항암효과를 위한 단백질 백신으로 개발되었다. 최근 보고된 연구에 따르면, GV1001이 항암제로써의 기능뿐만 아니라 세포 투과성 펩티드 기능과 항염증 효과를 가진다고 보고된 바 있다. ENO1은 해당과정에 관여하는 효소로 이것의 자극은 Con A에 의해 ENO1의 발현을 증가시킨 정상 말초혈액세포에서 뿐만 아니라 류마티스 관절염 환자에서 이미 많은 양의 ENO1을 발현하고 있는 단핵백혈구와 대식세포로부터의 염증성 사이토카인의 생성을 증가를 유도한다. 우리는 GV1001이 ENO1 자극

에 의해 유도되는 면역 반응에 미치는 효과를 규명하고자 하였다. 먼저, 정상 사람말초혈액을 Con A로 자극하여 ENO1의 발현을 증가시키고, 활성화된 정상 사람말초혈액을 ENO1에 대한 항체로 자극 하자 TNF-α, IL-1β 와 IL-6와 같은 염증성 사이토카인이 크게 증가하였다. 그리고 이와 같은 ENO1 자극으로 인한 염증성 사이토카인의 생성은 GV1001의 전 처리에 의해 감소하였다. 다음으로, ENO1이 이미 많이 발현되어 있는 류마티스 관절염환자의 말초혈액 세포에서도 ENO1을 자극했을 때 염증성 사이토카인이 상당량 생성되었고, 이 또한 GV1001 전 처리에 의해 효과적으로 감소함을 관찰할 수 있었다. 이에는 GV1001이 ENO1의 주요 신호전달인자인 p38 MAPK와 NF-κB을 억제함으로서 염증성 사이토카인의 감소를 유도함을 확인하였다. 따라서 GV1001이 환자의 말초혈액 세포에 ENO1의 발현 정도에는 영향을 미치지 않지만, ENO1에 의해 유도되는 염증 반응을 GV1001이 ENO1의 주요 신호전달기전을 억제시킴으로써 완화시킨다는 것을 알 수 있었다.

Keywords: 염증, GV1001, ENO1, 류마티스 관절염, p38 MAPK, NF-κB

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