의학석사 학위논문

Concanavalin A 염증 매개 모델과 류마티스 관절염에서 인터루킨-32 생성과 관련한 알파 에놀레이즈의 역할에 관한 연구

The role of α-enolase on the production of interleukin-32 in concanavalin A mediated inflammation and rheumatoid arthritis

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서울대학교 대학원

의학과 해부학 전공

이 준 명

ABSTRACT

The role of α-enolase on the production of interleukin-32 in concanavalin A mediated inflammation and rheumatoid arthritis

Junmyung Lee
Department of Anatomy
Seoul National University College of Medicine

Interleukin (IL)-32 is produced by T lymphocytes, natural killer cells, monocytes, and epithelial cells. It has recently been reported that it induces the production of pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, IL-1β, IL-6 and IL-8 and its expression is highly increased in the rheumatoid arthritis (RA) patients. α-Enolase (ENO1) is a glycolytic enzyme and the stimulation of ENO1 induces high level of pro-inflammatory cytokines from concanavalin A (Con A)-activated peripheral blood mononuclear cells (PBMCs) and macrophages from RA patients. In addition, there are many reports that anti-ENO1 antibody is correlated with the disease progression of RA. It implies that ENO1 could regulate IL-32 production during inflammation related with the pathogenesis of RA. Therefore, I performed whether ENO1 is involved in the production of IL-32 under inflammatory condition using Con A-activated

PBMCs and RA PBMCs. As a result, it was confirmed by RT-PCR and ELISA that the expression of IL-32 at mRNA and protein levels was increased by stimulation with anti-ENO1 mAb. To investigate the signaling pathway that is stimulated with anti-ENO1 mAb, I did immunoblotting for nuclear factor (NF)-κB and p38 mitogen activated protein kinase (MAPK) pathway. Phosphorylated p65 and p38 MAPK were increased by ENO1 stimulation. Finally, I confirmed that the increased IL-32 production by ENO1 stimulation was inhibited by the pre-treatment of BAY11-7082, SB203580. Taken together, these results suggest that ENO1 plays an important role in inflammation through the induction of IL-32 production that is mediated with the activation of NF-κB and p38 MAPK pathway.

Keywords: ENO1, IL-32, RA, Inflammation

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LIST OF ABBREVIATIONS

Con A: concanavalin A

ELISA: enzyme-linked immunosorbent assay

ENO: enolase

IBD: inflammatory bowel disease

IL: interleukin

LPS: lipopolysaccharide

NF-κB: nuclear factor-κB

NK cell: natural killer cell

MAPK: mitogen activated protein kinase

PBMCs: peripheral blood mononuclear cells

PBS: phosphate buffered saline

PBS-T: PBS containing 0.05% Tween 20

PGE_2: prostaglandin E2

PMA: phorbol myristate acetate

RA: rheumatoid arthritis

RT-qPCR: real-time reverse transcription-polymerase chain reaction

SD: standard deviation

TNF-α: tumor necrosis factor-α

TRAF6: tumor necrosis factor receptor-associated factor 6

INTRODUCTION

Interleukin (IL)-32, a described cytokine produced by T lymphocytes, natural killer (NK) cells, monocytes, and epithelial cells (1, 2). The genomic structure of IL-32 is organized into eight exons and located on human chromosome 16p13.3 and IL-32 exists in 6 isoforms; IL-32α, IL-32β, IL-32γ, IL-32δ, IL-32ε and IL-32ζ. IL-32γ is the full-length isoform without any exonic deletions and the most active isoform of the cytokine (1, 3). This cytokine induces other pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, IL-1, IL-6 and IL-8 by the activation of nuclear factor (NF)-κB and p38 mitogen activated protein kinase (MAPK) (4-6). IL-32 has recently been known to play an important role in inflammatory diseases such as inflammatory bowel diseases (IBD) and rheumatoid arthritis (RA). The expression level of IL-32 in the synovium of patients with RA highly increased and injection of IL-32 into the knee joints of mice resulted in joint swelling, infiltration of immune cells and cartilage damage (1, 2, 4).

Enolase (ENO) is a glycolytic enzyme that degrades 2-phosphogly -cerate to 2-phosphoenolpyruvate, in the last steps of the catabolic glycolytic pathway. In vertebrates, it exists as 3 isoforms; α-enolase (enolase-1 [ENO1]) is expressed in most tissues, whereas β-enolase (enolase-3 [ENO3]) is

predominantly found in muscle tissues and γ-enolase (enolase-2 [ENO2]) is found in neuron and neuroendocrine tissues (7, 8). ENO1 is normally expressed in the cytosol. But, ENO1 has been detected on the surface of hematopoietic cells; monocytes, T cells, B cells, neuronal cells and endothelial cells during pathological conditions such as inflammation, autoimmunity and malignancy (8-10). ENO1 expression is increased on cell surface by lipopolysaccharide (LPS), phorbol myristate acetate (PMA) and concanavalin (Con) A stimulation and it is involved in many functions for inflammatory responses (11-14). 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 (15-17). RA is a systemic autoimmune inflammatory disease that affects mostly multiple peripheral joints and includes synovial inflammation, pannus formation and subsequent bone destruction (18-21). Although the exact mechanisms that contribute to the pathogenesis are still unknown, it is well accepted that many cells such as T cells, B cells, fibroblast-like synoviocytes, antigen-presenting cells and their extensive production of pro-inflammatory mediators such as TNF-α, IL-1, IL-6, IL-15, IL-17 and IL-18 are involved (8, 14, 22).

In the previous study, Con A stimulation increased the expression of ENO1 on the surface of peripheral blood mononuclear cell (PBMC)s and resulted in increased the production of pro-inflammatory cytokines through NF-κB and p38 MAPK pathways (14). In addition, there are several reports regarding the role of IL-32 on the pathogenesis of RA (5), but it is not yet clarified whether ENO1 is involved in the production of IL-32, especially under inflammatory condition. Therefore, I investigated whether ENO1 is involved in the production of IL-32 under inflammatory condition using Con A-activated PBMCs and RA PBMCs and that is mediated with the activation of NF-κB and p38 MAPK pathways.

MATERIALS AND METHODS

Isolation of PBMCs Heparinized peripheral blood was collected from healthy volunteers and RA patients. Peripheral blood was mixed equal volume of phosphate buffered saline (PBS) and then peripheral blood mononuclear cells (PBMCs) were isolated with density gradient centrifuge using Ficoll-Paque™ PLUS (GE Healthcare Biosciences, Uppsala, Sweden). After centrifugation at 2000 rpm for 20 min, buffy coat was collected and washed twice with PBS and the residual red blood cells were lysed by red blood cell lysis buffer (Sigma-Aldrich, St. Louis, MO, USA) for 5 min. After cells were washed twice with PBS, cells were cultured in RPMI 1640 (WELGENE, Daegu, Korea) supplemented with 10% heat-inactivated fetal bovine serum (WELGENE), 100 U/ml of penicillin and 100 μg/ml streptomycin (WELGENE) at 37°C in a humidified incubator containing 5% CO_2.

Stimulation of PBMCs with Concanavalin A and anti-ENO1 mAb PBMCs from healthy volunteers were stimulated with Con A (Calbiochem, Darmstadt, Germany) (2 μg/ml) for 48 hrs. After Con A stimulation, PBMCs were washed twice with PBS. Con A activated PBMCs and PBMCs from RA patients were stimulated with anti-ENO1 mAb (1 μg/10^6 cells) at room

temperature with rotation for 1 hr. MOPC-21 (1 μg/10^6 cells; Sigma-Aldrich) was used as an isotype control antibody. After ENO1 stimulation, cells were transferred to 24-well plate and incubated at 37°C incubator.

Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-qPCR) To study the expression of IL-32γ in Con A activated PBMCs and PBMCs from RA patients, RT-qPCR was performed. Cells (2×10^6) were harvested at 6 hrs after ENO1 stimulation. Total RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA, USA) and complementary DNA was synthesized using 1 μg of total RNA with Reverse Transcription System (Promega, Madison, WI, USA). The PCR was conducted using a SYBR Green PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA). The primer used for the RT-qPCR was as follows: 5′-AGGCCCGAATGGTAATGCT-3′ (forward) and 5′-CCACAGTGT CCTCAGTGTCACA-3’ (reverse) for IL-32γ (85 bp); 5′-GGTGGTCTCCTCT GACTTCA-3′ (forward) and 5′-GTTGCTGTAGCCAAATTCGT-3’ (reverse) for GAPDH (127 bp). Typical profile times were the initial step, 95°C for 10 min followed by a second step at 95°C for 15 s and 60°C for 30 s for 40 cycles with a melting cure analysis. The level of target mRNA was normalized to the level of the GAPDH and compared with the control. Data were analyzed using the ΔΔCT method.

Enzyme-Linked Immunosorbent Assay (ELISA) After Con A activation, cells (5×10^6) were stimulated with anti-ENO1 mAb (1 μg/10^6 cells) at room temperature with rotation for 1 hr and incubated for 48 hrs. The concentration of IL-32, TNF-α and IL-6 in culture supernatant were measured by ELISA kit (R&D systems, Minneapolis, MN, USA: IL-32, TNF-α, Biolegend, San Diego, CA, USA: IL-6) according to the manufacturer's instruction. The relative absorbance was measured at 450 nm using the SoftmaxPro software (Molecular Devices, Sunnyvale, CA, USA).

Inhibitor study for signaling pathway Specific inhibitors for NF-κB (Bay 11-7082) and p38 MAPK (SB203580) were purchased from Sigma-Aldrich. These inhibitors were used to identify the signaling pathways involved in the inductions of cytokines by ENO1 stimulation with anti-ENO1 mAb. Con A activated PBMCs and PBMCs from RA patients (5×10^6) were pretreated with DMSO (vehicle control) or inhibitors (Bay 11-7082: 2.5 μM, SB203580: 40 μM) for 1 hr and washed twice with PBS. Then cells were stimulated with anti-ENO1 mAb (1 μg/10^6 cells) at room temperature with rotation for 1 hr.

Western blot analysis After ENO1 stimulation with anti-ENO1 mAb, cells (2×10^6) were lysed and proteins extracted in a 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 and phosphatase inhibitor cocktails (Sigma-Aldrich). An equal amount of protein at 30 μg per sample was dissolved in a 10% polyacrylamide-SDS gel with 100 V for 4 hrs and transferred onto nitrocellulose membrane. Blocking was performed at room temperature for 1 hr with 5% non-fat milk in PBS containing 0.05 % Tween 20 (PBS-T). The blocked membrane was incubated with anti-p65 Ab (1:200; Santa Cruz Biotechnology, Santa cruz, CA, USA), anti-phospho-p65 Ab (1:500; Cell signaling, Boston, MA, USA), anti-p38 MAPK Ab, anti-phospho-p38 MAPK Ab, anti-β-actin Ab (1:1000; Cell signaling) and TRAF6 (1:2000; Abcam, Cambridge, UK) at 4^{o}C overnight. After washing for 3 times with PBS-T, membrane was incubated with horse radish peroxidase (HRP)-conjugated anti-rabbit IgG secondary Ab (1:5000; Cell signaling) for anti-p65, anti-phospho-p65, anti-p38, anti-phospho-p38, TRAF6 and HRP-conjugated anti-mouse IgG secondary Ab for β-actin at room temperature for 1 hr. The membrane was then washed for 3 times with PBS-T and the immunoreactive proteins were visualized with the electrochemical luminescence (ECL) detection system (EZ-Western Lumi La; Dogen, Seoul, Korea). The bands were analyzed for their density using Image J software (NIH, Bethesda, MD, USA). Results were expressed as relative intensity and each band was adjusted to that of β-actin.

Statistical analysis Data are presented as mean ± SDs. Unpaired two-tailed *t* test was used to compare two groups. Statistical analysis was carried out using GraphPad InStat version 5.01 (GraphPad Software, La Jolla, CA, USA). The *p* values <0.05 were considered statistically significant.

RESULTS

1. IL-32γ expression at transcriptional level in Con A-activated PBMCs by ENO1 stimulation

To investigate whether IL-32γ mRNA expression is increased in Con A-activated PBMCs by ENO1 stimulation, RT-qPCR was performed. After PBMCs ($2x10^6$) were obtained from healthy individuals and activated with Con A (2 μg/ml) for 48 hrs, activated PBMCs were stimulated with anti-ENO1 mAb (1 μg/10^6 cells) for 1 hr. As shown in Fig. 1, IL-32γ mRNA expression by ENO1 stimulation was 1.4 folds increased than in those of control without stimulation. This result indicates that IL-32γ mRNA expression is increased by ENO1 stimulation from Con A-activated PBMCs.

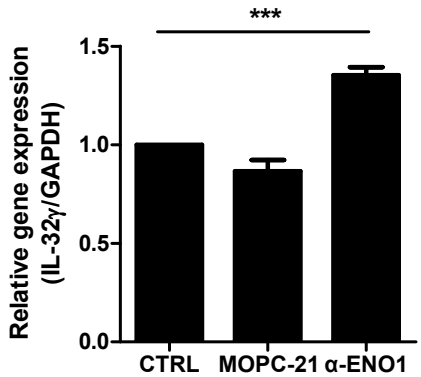

Fig. 1 IL-32γ mRNA expression by ENO1 stimulation from Con A-activated PBMCs

Isolated PBMCs ($2x10^6$) from normal individuals were stimulated with Con A (2 μg/ml) for 24 hrs. Activated PBMCs were collected at 6 hrs after stimulation with anti-ENO1 mAb (1 μg/10^6 cells) for 1 hr. MOPC-21 was used as an isotype control. And then, total RNA was extracted and cDNA was made. RT-qPCR was performed by using the specific probe for IL-32γ as described in *Materials and Methods*. Results were expressed as relative intensity and each group was adjusted to that of GAPDH. Each sample is in triplicates and data are presented as the mean ± SD. ***$p<0.001$

2. IL-32 expression at translational level in Con A-activated PBMCs by ENO1 stimulation

Based on the report by Bae *et al*., ENO1 stimulation increases the production of pro-inflammatory cytokines such as IL-1α/β, IL-6, IL-18 and TNF-α, from RA PBMCs (14). However, it is still not reported whether IL-32 production is increased by ENO1 stimulation. For this reason, I tried to find whether the level of IL-32 was increased by ENO1 stimulation with anti-ENO1 mAb from Con A-activated PBMCs. After Con A-activated PBMCs ($5x10^6$) were stimulated with anti-ENO1 mAb for 1 hr and then incubated for 48 hrs. The levels of IL-32 and other pro-inflammatory, IL-6 and TNF-α were measured by ELISA. As I expected, the production of IL-32 was also increased by ENO1 stimulation from Con A-activated PBMCs like the increase of IL-6 and TNF-α (Fig. 2). Therefore, IL-32 production is increased by ENO1 stimulation from Con A-activated PBMCs.

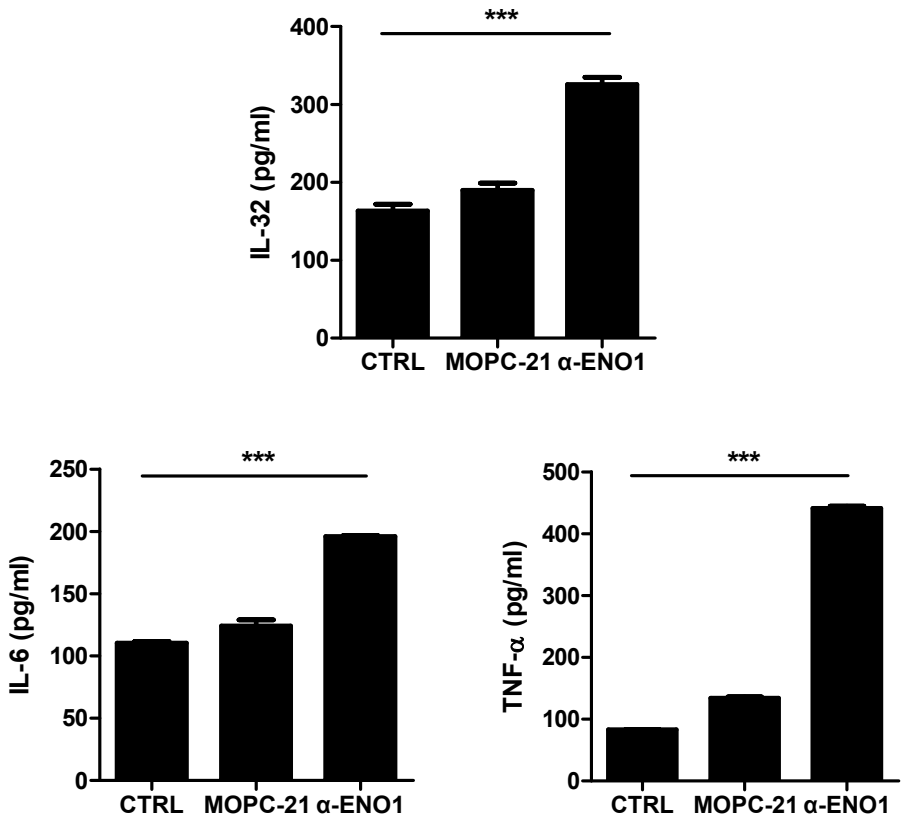

Fig. 2 IL-32 production by ENO1 stimulation from Con A-activated PBMCs

After Con A activation, PBMCs (5×10^6) were stimulated with anti-ENO1 mAb (1 μg/10^6 cells) at room temperature with rotation for 1 hr and incubated for 48 hrs. MOPC-21 was used as an isotype control. Culture media was collected and centrifuged at 600g for 10 min. The supernatant was harvested and the production of IL-32, IL-6 and TNF-α was determined by ELISA. Data are presented as the mean ± SD. ***$p<0.001$

3. IL-32 production was increased in Con A-activated PBMCs by ENO1 stimulation via the activation of NF-κB and p38 MAPK

It has been reported that ENO1 increases the production of pro-inflammatory mediators through the activation of NF-κB and p38 MAPK pathway in Con A-activated PBMCs and RA PBMCs (14). I also confirmed the activation of NF-κB and p38 MAPK pathway by Western blot analysis, in a time dependent manner of ENO1 stimulation. As shown in Fig. 3, at 30 min after ENO1 stimulation, the phosphorylation of p65 was increased and peaked at 120 min after stimulation. In case of p38 MAPK, the phosphorylation of p38 MAPK increases at 60 min after stimulation (Fig. 3). Next, I investigated whether these the activation of NF-κB and p38 MAPK are involved in ENO1-induced IL-32 production in Con A-activated PBMCs. Con A-activated PBMCs were pre-treated with BAY11-7082 (specific inhibitor for NF-κB) and SB203580 (specific inhibitor for p38 MAPK) for 1 hr, and then stimulated with anti-ENO1 mAb. After incubating for 48 hrs, I examined the changes in the production of IL-32 were measured by ELISA. Like the suppression of IL-6 and TNF-α production by the pre-treatment of BAY11-7082 and SB203580, I found that ENO1-induced IL-32 production was also suppressed by the treatment of BAY11-7082 and SB203580 (Fig. 4). Taken together, it suggests that ENO1 increases the production of IL-32 via the activation of NF-κB and p38 MAPK.

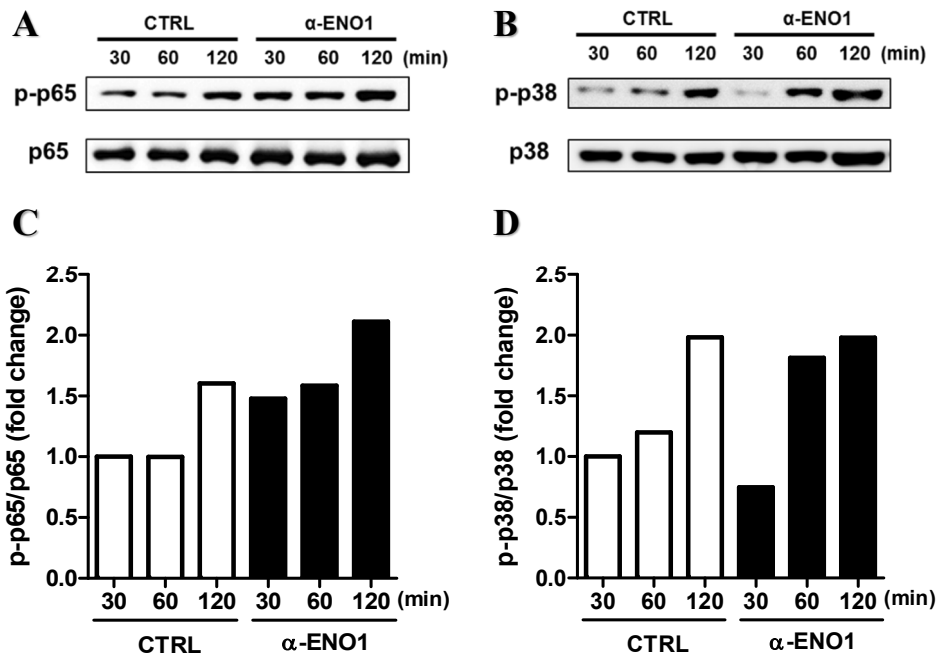

Fig. 3 Increase of the phosphorylation of NF-κB and p38 MAPK in Con A-activated PBMCs by ENO1 stimulation

PBMCs were incubated with Con A (2 μg/ml) for 48 hrs and stimulated with anti-ENO1 mAb (1 μg/10^6 cells) for 30, 60 and 120 min. Cells were lysed and protein was extracted for western blot analysis as described in *Materials and Methods*. (A and B) The expression of p65, p38 and its phosphorylation was examined by western blot analysis. (C and D) Densitometry analysis was performed and it was represented as fold change of phosphorylated form to total form, p-p65/p65 and p-p38/p38.

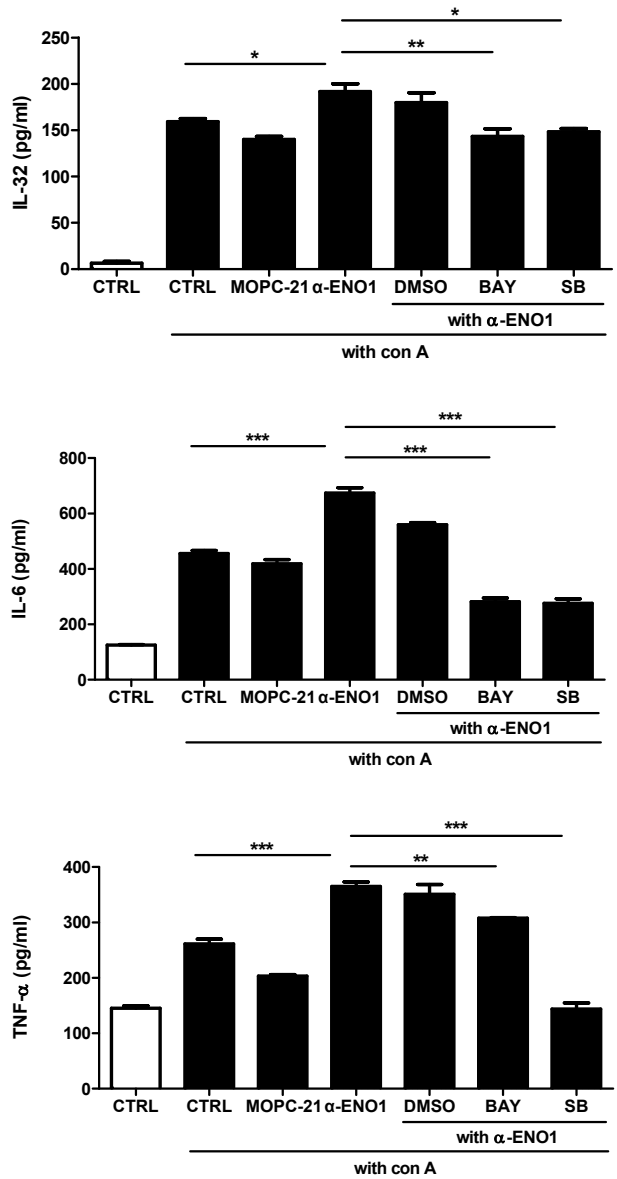

Fig. 4 Inhibition of increased IL-32 production by ENO1 stimulation with the pre-treatment of BAY11-7082 and SB203580

After Con A activation, PBMCs (5×10^6) were pre-treated with DMSO (vehicle control), BAY11-7082 (2.5 μM) and SB203580 (40 μM) for 1 hr and

then stimulated with anti-ENO1 mAb (1 μg/10^6 cells) for 1 hr. MOPC-21 was used as an isotype control. After incubating for 48 hrs, culture supernatant was collected and centrifuged at 600g for 10 min and then the amounts of IL-32, IL-6 and TNF-α were examined by ELISA. Data are presented as the mean ± SD. $^{*}p<0.005$, $^{**}p<0.01$, $^{***}p<0.001$

4. IL-32γ mRNA expression and production in RA PBMCs by ENO1 stimulation

It is known that Con A-activated PBMCs is widely used as model of several kinds of inflammatory diseases including rheumatoid arthritis, which is a systemic autoimmune disease and characterized by synovial inflammation and destruction of bone and cartilage (14, 18-21). As shown in Fig. 1 and 2, IL-32γ mRNA expression and its protein production were increased by ENO1 stimulation from Con A-activated PBMCs. Therefore, I did the experiment by using RA PBMCs to confirm the results shown in the experiments using Con A-activated PBMCs. After PBMCs were obtained from RA patients and stimulated with anti-ENO1 mAb for 1 hr and incubated for 6 or 48 hrs, and then I measured the expression of IL-32 at the mRNA and its protein production by RT-qPCR and ELSIA, respectively. As shown in Fig 5A, IL-32γ mRNA expression by ENO1 stimulation increased 1.3-fold than those of control. And production of IL-32 by ENO1 stimulation was also increased by ENO1 stimulation from RA PBMCs (Fig. 5B). These results indicate that IL-32γ mRNA expression and production are increased by ENO1 stimulation from RA PBMCs.

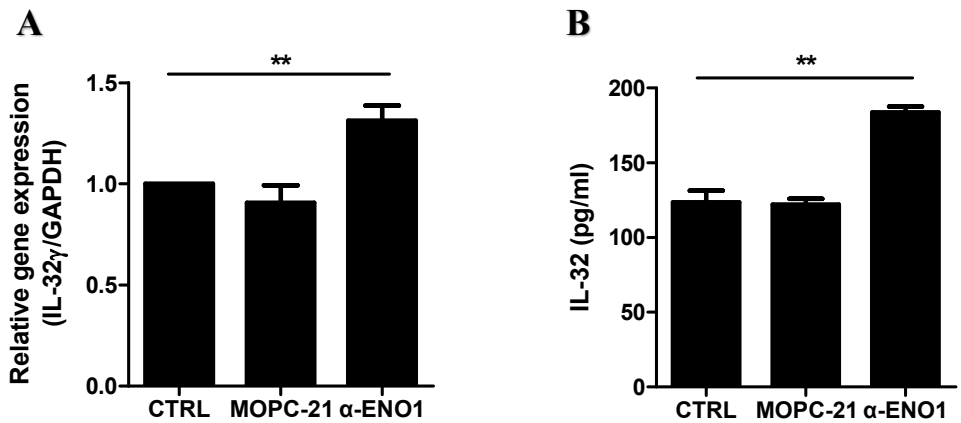

Fig. 5 Increase of IL-32γ mRNA expression and production in RA PBMCs by ENO1 stimulation

(A) PBMCs ($2x10^6$) from RA patients were stimulated with anti-ENO1 mAb (1 μg/10^6 cells) or MOPC-21 (isotype control) for 1 hr. After incubating for another 6 hrs, total RNA was extracted and cDNA was made. RT-qPCR was performed by using the specific primer for IL-32γ as described in *Materials and Methods*. Results were expressed as relative intensity and each group was adjusted to that of GAPDH. Each sample is in triplicates and data are presented as the mean ± SD. (B) PBMCs (5×10^6) from RA patients were stimulated with anti-ENO1 mAb (1 μg/10^6 cells). MOPC-21 was used as an isotype control. After 48 hrs, the culture supernatant of stimulated PBMCs was collected and the production of IL-32 was measured by ELISA. Data are presented as the mean ± SD. $^{**}p<0.01$

5. IL-32 production was increased in RA PBMCs by ENO1 stimulation via the activation of NF-κB and p38 MAPK

I already confirmed that ENO1-induced IL-32 production was increased from Con A-activated PBMCs through the activation of NF-κB and p38 MAPK (Fig. 3 and 4). Therefore, I also examined whether the activation of NF-κB and p38 MAPK are involved in ENO1-induced IL-32 production in RA PBMCs. In accordance with results shown in Fig. 3 and 4, after ENO1 stimulation with anti-ENO1 mAb for 30, 60 and 120 min, phosphorylation of p65 and p38 MAPK was found to increase in PBMCs from RA patients (Fig. 7). Next, RA PBMCs were pre-treated with BAY11-7082 and SB203580 for 1 hr, and then stimulated with anti-ENO1 mAb. After 48 hrs, the culture supernatants were collected and the concentration of IL-32 was determined by ELISA. As I expected, ENO1-induced IL-32 production was suppressed by BAY11-7082 and SB203580 (Fig. 8). This result indicates that ENO1 also increases the production of IL-32 from RA PBMCs through NF-κB and p38 MAPK.

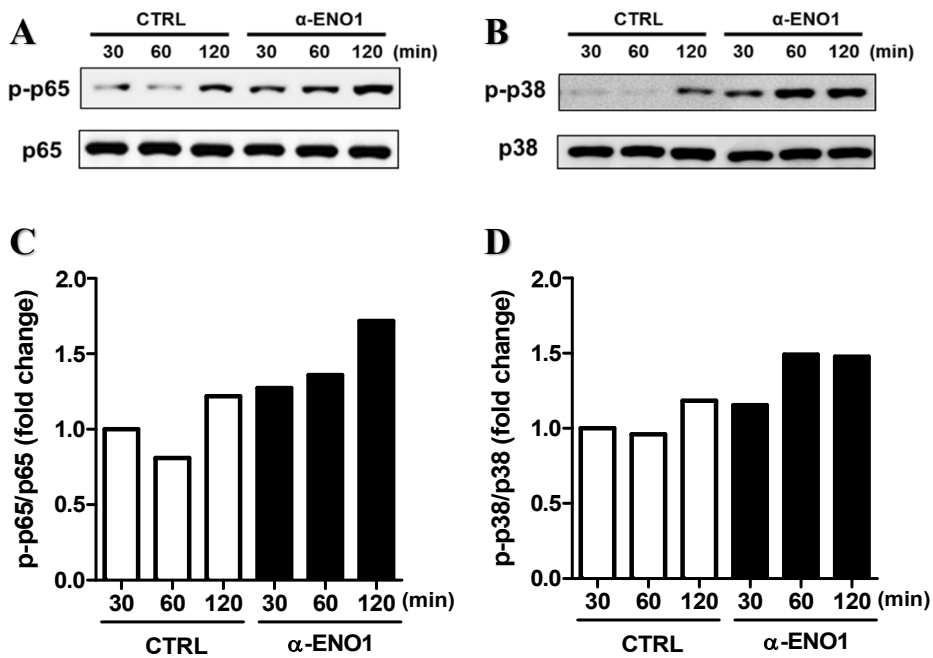

Fig.6 Increase of the phosphorylation of NF-κB and p38 MAPK in RA PBMCs by ENO1 stimulation

RA PBMCs were stimulated with anti-ENO1 mAb (1 μg/10^6 cells) for 30, 60 and 120 min. Cells were lysed and protein was extracted for western blot analysis as described in *Materials and Methods*. (A and B) The expression of p65, p38 and its phosphorylation was examined by western blot analysis. (C and D) Densitometry analysis was performed and it was represented as fold change of phosphorylated form to total form, p-p65/p65 and p-p38/p38.

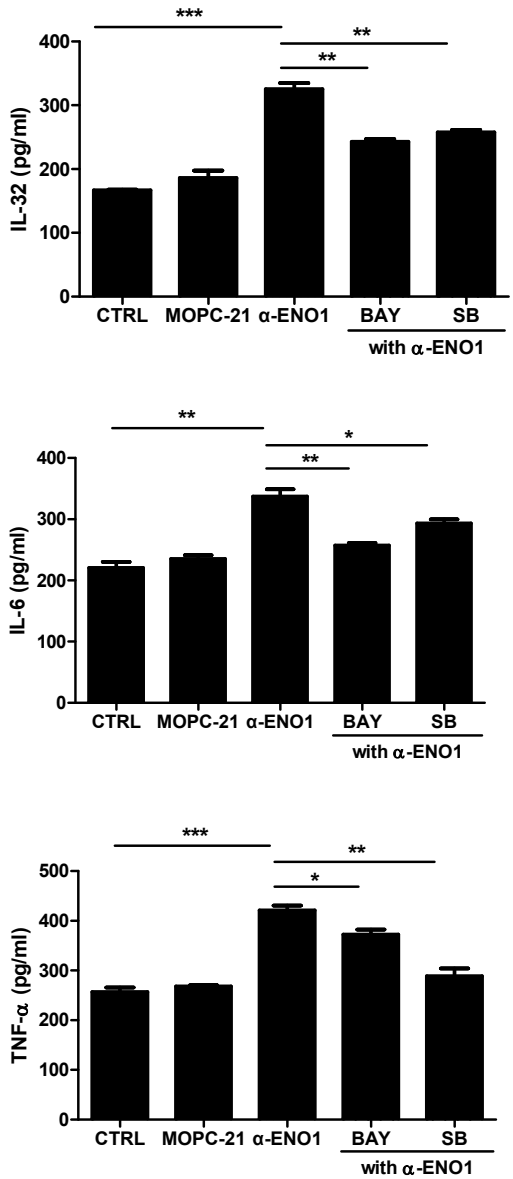

Fig. 7 Inhibition of increased IL-32 production by ENO1 stimulation with the pre-treatment of BAY11-7082 and SB203580 in RA PBMCs

PBMCs from RA patients were pre-treated with DMSO (vehicle control), BAY11-7082 (2.5 μM) and SB203580 (40 μM) for 1 hr and then

stimulated with anti-ENO1 mAb (1 μg/10^6 cells) or MOPC-21 (isotype control) for 1 hr. After 48 hrs, culture supernatant was collected and centrifuged at 600g for 10 min, and then the amounts of IL-32, IL-6 and TNF-α were examined by ELISA. Data are presented as the mean ± SD. $*p<0.005$, $**p<0.01$, $***p<0.001$

DISCUSSION

It is known that inflammation is pivotal process for immune protection through the production of inflammatory mediators including inflammatory cytokines and Prostaglandin E2 (PGE_2). However, it might cause fatal effect, when it is uncontrolled. Therefore, there are many studies regarding the down-regulation of inflammatory mediators as well as the investigation of inflammatory stimulators. It is recently reported that ENO1 and IL-32 play pathogenic roles in a variety of inflammatory diseases (4-6, 15-17). However, it is not yet clarified whether ENO1 is involved in the production of IL-32 under inflammatory condition.

As mentioned above, IL-32 is a novel pro-inflammatory cytokine that is induced by TNF-α, IL-1α, IL-6 and IL-8. Additionally, it stimulates production of various inflammatory mediators through NF-κB and p38 MAPK pathway and has an important role in progression of various inflammatory disorders such as RA and IBD (2, 5, 6). ENO1, an essential glycolytic enzyme, is located in the cytosol in a normal condition, but translocated to the cell membrane in pathological conditions such as inflammation and autoimmunity (8-10). When the translocated ENO1 from cytosol to cell surface is stimulated, increases the production of pro-inflammatory mediators through the activation

of NF-κB and p38 MAPK pathway in Con A-activated PBMCs and RA PBMCs (14). These signaling pathways play important roles in pathogenesis of RA. NF-κB pathway forms a positive regulatory that may amplify and maintain the process of RA (24). Also, p38 MAPK pathway in RA contributes to the up-regulation of pro-inflammatory cytokines and chemokines (25).

I have shown the increased IL-32γ expressions at transcriptional and production in Con A-activated PBMCs and RA PBMCs by ENO1 stimulation (Fig. 1, 2 and 5). Based on reports about the role of IL-32 in several kinds of inflammatory diseases, it suggests that ENO1 might be a important stimulator on the production of IL-32. Therefore, IL-32 mediated inflammatory responses could be effectively regulated by the inhibition of ENO1 activity. As shown in Fig. 3 and 6, ENO1 stimulation significantly increases the activation of NF-κB and p38 MAPK. In addition, IL-32 expressions at transcriptional and translational level were increased by ENO1 stimulation from Con A-activated PBMCs and RA PBMCs via the activation of NF-κB and p38 MAPK pathway. (Fig 4. and 7). I also confirmed that the production of IL-6 and TNF-α by ENO1 stimulation were also decreased after treatment of BAY11-7082 and SB203580. It means that NF-κB and p38 MAPK play a crucial role on the production of mediators, especially IL-32, during inflammatory process that is induced by ENO1.

In relation with NF-κB activation, it has been reported that tumor necrosis factor receptor-associated factor (TRAF) 6 is a key adapter molecule in regulating various signaling pathways of inflammatory response and a diverse array of physiological processes, including adaptive immunity, innate immunity and bone metabolism (26, 27). Since it also influences the differentiation of osteoclasts through several pathways, including NF-κB and p38 MAPK (28), the activation of TRAF6 was investigated in my experiment. As I expected, the activation of TRAF6 was definitely increased after ENO1 stimulation (data not shown), but it should be further investigated whether TRAF6 is closely related with IL-32 production by ENO1 stimulation.

The most well-known substance involved in the migration of inflammatory cells to inflammatory sites through the ENO1 stimulation and the production of inflammatory substances is plasminogen (29). According to report by Wygrecka *et al*., plasminogen is activated by the urokinase plasminogen activator and then stimulates ENO1 on the surface of inflammatory cells to induce inflammatory cell migration to the inflammatory site in pneumonia (11). It means that the inflammatory response can be controlled through the regulation of plasminogen activity, and many studies related to this have been conducted. However, it is not yet known whether plasminogen is involved in IL-32 production during the inflammatory response.

Therefore, additional research about the regulation of IL-32 production by the regulation of plasminogen activity is also needed.

Studies on the regulation of IL-32 production through the interaction between plasminogen and ENO1 are important not only for inflammation but also for tumorigenesis. In fact, studies on increased expression of ENO1 on tumor cell surfaces have already been reported (30). Moreover, ENO1 promotes cell proliferation, migration, invasion, and tumorigenesis in non-small cell lung cancer (31). Recently, there have been many reports that IL-32 plays an important role in tumorigenesis. Based on the report by Nishida *et al*, IL-32 is highly expressed in pancreatic cancer in comparison with normal pancreatic cells, and IL-32 has pro-cancer effects that inhibit apoptosis through enhancement of anti-apoptotic proteins and stimulates DNA synthesis in proliferation of pancreatic cancer cells (32). In gastric cancer, IL-32 is also highly expressed in cancerous tissue and serum of patients (33) and increases human gastric cancer cell invasion associated with tumor progression and metastasis (34).

In conclusion, this study suggests that the regulation of IL-32 production through the regulation of ENO1 activity is an effective way to control inflammatory response in several kinds of disease including RA.

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국 문 초 록

Concanavalin A 염증 매개 모델과 류마티스 관절염에서 인터루킨-32 생성과 관련한 알파 에놀레이즈의 역할에 관한 연구

서울대학교 의과대학
해부학 전공 이 준 명

인터루킨-32는 최근에 발견된 사이토카인으로 주로 T 림프구, 자연살해세포, 단핵구, 상피세포에서 분비되어 염증 반응을 촉진한다. 또한, 인터루킨-32는 종양괴사인자-α (TNF-α), 인터루킨-1β, -6, -8과 같은 염증성 사이토카인의 생성을 유도하는데, 주로 염증성 장 질환이나 류마티스 관절염 환자에서 IL-32의 발현이 높게 증가되어 있다고 알려져 있다. 알파 에놀레이즈(ENO1)는 해당과정에 관여하는 효소로 다중 활성 자극 인자인 Concanavalin (Con) A로 자극한 사람 말초혈액세포와 류마티스 관절염 환자의 말초혈액세포에서 염증성 사이토카인의 증가를 유도한다고 알려져 있다. 다양한 염증성 질환과 류마티스

관절염에서 알파 에놀레이즈에 대한 항체의 증가가 질병의 진행과 관련되어 있다는 연구들이 많이 보고되어 있으나, 이러한 염증성 질환에서 알파 에놀레이즈가 IL-32의 생성과 직접적으로 관련이 있는지에 대해서는 아직까지 알려진 바 없다. 따라서, 본 연구에서는 알파 에놀레이즈가 Con A에 의한 염증 모델과 류마티스 관절염에서 염증 반응 동안에 IL-32의 생성 증가에 관여하는 지에 대하여 규명하고자 하였다. 먼저, Con A로 자극한 말초혈액세포와 류마티스 관절염 환자의 말초혈액세포에서 알파 에놀레이즈의 자극에 의해 mRNA와 단백질 수준에서 인터루킨-32의 증가를 확인하였다. 다음으로 알파 에놀레이즈의 자극과 관련된 신호전달 경로를 확인한 결과, Con A로 자극한 말초혈액세포와 류마티스 관절염 환자의 말초혈액세포를 알파 에놀레이즈로 자극하였을 경우 NF-κB와 p38 MAPK의 활성이 증가됨을 확인하였다. NF-κB와 p38 MAPK의 활성이 알파 에놀레이즈의 자극에 의한 인터루킨-32 생성과 연관성이 있는 지의 여부를 NF-κB와 p38 MAPK의 억제제 (BAY11-7082, SB203580)를 처리를 통하여 확인 한 결과, 알파 에놀레이즈의 자극에 의해 증가된 인터루킨-32의 생성이

BAY11-7082와 SB203580를 처리하자 감소하는 것을 확인하였다. 알파 에놀레이즈는 Con A에 의한 염증 모델과 류마티스 관절염에서 NF-κB와 p38 MAPK의 활성을 통하여 인터루킨-32의 생성을 증가시킨다는 것을 알 수 있었다.

주요어: 알파 에놀레이즈, 인터루킨-32, 류마티스 관절염, 염증

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