약학석사 학위논문

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서울대학교 융합과학기술대학원

분자의학 및 바이오제약학과 의약생명과학전공

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지도교수 김 성 훈
이 논문을 약학석사 학위논문으로 제출함

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서울대학교 융합과학기술대학원
분자의학 및 바이오제약학과
의약생명과학 전공
김 해 준

김해준의 약학석사 학위논문을 인준함
2016 년 2 월

위 원 장 (인)

부 위 원 장 (인)

위 원 (인)

ABSTRACT

ARS is an enzyme that pairs tRNA with each amino acid of its own. Among the 20 different amino acids eukaryotes carry, threonyl-tRNA synthetase (TRS) delivers Threonine to tRNA to regulate protein synthesis. However, there is not much of a study done on functions of TRS other than conventional features. It is currently known that TRS is secreted by the external stimulus such as VEGF and TNFα. This mechanism causes metastasis and angiogenesis of tumor, but it is not well studied how secreted TRS functions in this circumstances. Inflammatory cytokines are activated by extracellular signaling and then activated cytokines are secreted through the cell membrane. I expected secreted TRS can do a role of inflammatory function. So, I expressed and purified recombinant human TRS in *E. coli*. Responses of TRS were studied with the purified TRS on various cell lines. Consequently, I found that recombinant TRS can stimulate important cytokines and chemokines such as IL-6 and IL-8, respectively. On this result, for confirming cytokine activities of TRS, TRS plays an important role in inducing the inflammatory immune response. The present study suggested that TRS can be developed as a novel biomarker targeting newly discovered immune responses and inflammatory diseases.

Keywords : secreted Threonyl-tRNA Synthetase (TRS), Inflammatory cytokine,

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INTRODUCTION

Aminoacyl-tRNA synthetases (ARSs) are enzymes that charge amino acid to their cognate tRNA, important step for the protein synthesis. Although ARSs are well known by catalytic enzyme function, studies are unveiling other functions of some ARSs. ARSs are related with not only protein synthesis but also immune response and transcription factors (1). Also recent studies show that some ARSs are connected to angiogenesis (2). Therefore, ARSs expression levels are associated with many diseases including autoimmune disease and cancers (1, 3). However, those ARSs' other functions are not fully reported.

As secondary functions, some ARSs are secreted in the response to inflammation cytokines and affects cell signaling pathway (4, 5). For example, tyrosyl-tRNA synthetase (YRS) and tryptophanyl-tRNA synthetase (WRS) are secreted by stimulation of TNFα and interferon, respectively. Secreted YRS are cleaved and working as an angiogenic factor while secreted splice variant of WRS inhibits angiogenesis (6-8). TRS is one of the ARSs that are secreted into extracellular space through the transmembrane. The enzyme activity of TRS is well known with structure based study (9, 10). The previous study found that TRS is stimulated with TNFα, VEFG signaling, effects cell migration and angiogenesis (2). This can be evidence that TRS can be one of the important factors to cancer progression. Also, there are reports that anti-TRS PL-7 (auto-TRS antibody) are related to the autoimmune diseases such as myositis and interstitial lung disease (ILD) which are concerned with the relatively high expression of auto-antibody (11, 12). Although, some studies with TRS-related disease (13), unfortunately, TRS mechanisms of cytokines activities are not well studied yet.

Immune cytokine levels are changed by stimulating

extracellular signaling. For example, IL-8, IL-6, and TNFα are typical proinflammatory cytokine and chemokine for immune responses (14, 15). Those inflammatory molecules are important in immune response such as chemokines activities and differentiation of immune cells (16, 17), respectively. They are secreted and bind to receptors on cell surface membrane transmitting cell signaling. Thus, the variation of cytokines can be connected with immune diseases and also be charged for a marker of immune response. Secreted TRS is expected to take a role as a cytokine for immune response.

In this study, I confirmed roles of TRS as a cytokine function *in vitro* in various cell lines and *in vivo* mouse study. The present study suggests the novel function of TRS as a cytokine activity.

ABBREVIATIONS LIST

ARS: Aminoacyl-tRNA synthetase

ELISA: Enzyme-Linked ImmunoSorbent Assay

FPLC: Fast Protein Liquid Chromatography

IL-: Interleukin -

LPS: Lipopolysaccharide

PBMC: Peripheral Blood Mononuclear Cell

PCR: Polymerase Chain Reaction (PCR)

TNFα: Tumor Necrosis Factor α

TRS: Threonyl-tRNA synthetase

Keywords: ARS, TRS, Interleukin

MATERIALS AND METHODS

1. Cell culture

HUVEC and J774a.1 cells were cultured in DMEM medium (with 4mM L-Glutamine, 4500 mg/L Glucose, and Sodium Pyruvate, Hyclone, USA). THP1, RAW264.7 were cultured in RPMI-1640 medium (with 25mM HEPES and L-Glutamine, Hyclone) with 10% FBS and antibiotics. HMC1 cells were cultured in Iscove's modified Dulbecco's medium (IMDM) medium enrich with 10% FBS and antibiotics. A549 cells were cultured in Ham's F-12K medium with 10% fetal bovine serum (FBS, Hyclone) and 100 μg/mL of penicillin and streptomycin at 37 ℃ in 5% CO_2 incubator.

2. Recombinant human Threonyl-tRNA synthetase production

The human sequence of TRS was inserted into pET21a vector (Novagen, USA), which contains 6 histidine tag (His-tag) at C-terminus. BL21- codon plus (DE3)-RIPL cell are used for expression of human TRS. Cells were cultured in LB broth with 100 mg/ml ampicillin. The culture was incubated at 37℃ and TRS induced by 0.5 mM IPTG, 16 hrs at 18 ℃. The cells were harvested by centrifugation at 9500 g for 20 min.

Cells were lysed by sonication in PBS buffer with 15 mM imidazole. Proteins were harvested by centrifuge at 10000 rpm for 10 min at 4 ℃. Repeated centrifuge with same conditions with the supernatant.

Purification of TRS was performed by Ni-NTA resin. Supernatants were loaded onto Ni-NTA column and washed with lysis buffer (PBS buffer +15 mM imidazole) and washed with 50 mM imidazole and Triton X-114 0.1% (Sigma, USA) in PBS 50 mL. NI-NTA resin re-washed with 50mM Imidazole in PBS 50 mL. His-tagged TRS were eluted using 250 mM imidazole in PBS buffer.

The proteins were further purified by anion exchange. The recombinant protein was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and confirmed by silver staining.

3. Western blotting

THP1 cells ($1x10^6$) were seeded in a 6 well plate. His^6-tagged recombinant TRS, purified by anion exchange were added 100 ng/mL. Cells were harvested and lysed with cell lysis buffer (50mM Tris-HCL pH 7.4, 0.5 % Triton X-100, 5mM EDTA, 10% glycerol, 150 mM $NaCl_2$, and proteases inhibitor). After centrifugation, total proteins of supernatant were quantified by Bradford assay (BioRad, Cat. 500-0006, Hercules, California), and samples were loaded on 10% SDS-PAGE gel and electrophoresed. Separated proteins were transferred to PVDF (polyvinylidene fluoride) membrane (Millipore). The membranes were blocked with 5% BSA/TBST. The membranes were probed with anti-phospho-p44/42 MAPK, anti-phospho-JNK, anti-phospho-p38 MAPK antibodies from R&D Systems (Minneapolis, MN), and anti-IKBα form Santa Cruz Biotechnology (PasoRobles, CA).

4. Cytokine assays

Cells were seeded 5 x 10^4 ~ 1 x 10^6 per well on 96 well plate and added TRS with various concentrations for overnight. Capture cytokines were coated on ELISA plate (SPL life science, Gyeonggi-do, Korea). Nonspecific protein binding sites were blocked with 2% BSA in PBS for 2hrs at RT. The plates were washed with PBS containing 0.1% Tween-20 (PBS-T). TRS treated culture supernatants (100 mL) added to the plate for 2h. Plate washed with 200 μl of PBS-T at 3 times. Detection antibodies added each well and incubated for 2hrs. After washing the wells

were incubated for 40 min with HRP-conjugated antibodies against for (detection antibodies). Tetramethyl-benzidine liquid substrate (Sigma-Aldrich, ST. Louis, MO), and then stopped with 1 M H_2SO_4. The absorbance at 450nm was measured using an ELISA plate reader (Molecular Devices, Sunnyvale, CA).

5. Bioassay involving primary human and mouse cells

Human PBMCs were isolated by density centrifugation of blood over ficoll-Paque^{TM} PLUS (GE Healthcare Life Sciences, Piscataway, NJ). PBMCs were washed twice with saline (0.9% sodium chloride) and resuspended in culture medium (RPMI 1640, Hyclone).

Cells were seeded in a 100 ml volume in 96-well plates, which were then incubated with either 100 ml of either culture medium (control) or presence of TRS with culture medium. Human/mouse IL-6 or IL-8 levels were measured in the cell culture supernatants by using an ELISA kit (R&D systems, Minneapolis, MN). For measuring tissue cytokine levels, tissues of TRS treated mice were homogenized in cell lysis buffer containing 1% Triton X-100, and the IL-6 levels were measured as described above.

RESULT

Expression and purification of recombinant human threonyl-tRNA synthetase

We used pET21a plasmid vector for TRS expression. PCR productions of TRS and pET21a vector were confirmed by agarose gel electrophoresis. (**Figure 1A**) Nde1, Xho1 restriction enzymes cut TRS for inserting into pET21a plasmid vector (**Figure 1B**). pET21a has His^6 tag that fused with TRS for purification. Human TRS/pET21a plasmid vector is transformed into *E. coli* codon+ strain for expression of recombinant TRS. Ni-NTA resin is used in the primary purification step. During expression step, Soluble and insoluble TRS proteins were analyzed for confirming the expression of human TRS by western blot. (**Figure 2A**) For the analysis of His^6-tag fused TRS protein, 10% SDS-PAGE gel was performed and detected by Coomassie blue staining. (**Figure 2B**)

The second step purification of TRS with anion exchange chromatography.

Anion exchange chromatography was carried out for the second step purification. Protein was run through an anion column. Major peaks of recombinant TRS protein was separated at A2~A8 fractions as unbound forms as well as B15~B10 fractions for bound forms (**Figure 3A**). I collected each eluted fractions and the second step purified recombinant protein was visualized by silver staining. After 2^{nd} purification, non-specific bands are reduced than after 1^{st} purification with Ni-NTA resin (**Figure 3B**). The anion exchange chromatography eluted fractions containing recombinant TRS as determined by silver staining were pooled and quantified by comparing to BSA. (**Figure 3C**)

A distinct biological activity of TRS in THP1 cells

Whether secreted TRS have distinct biological activities remain unclear. Purified TRS by anion exchanged have 2 forms that are unbound and bound forms dependent on charges of proteins. I tested both of 2 forms and those showed biological activity in THP1 cells. However, I decided to use unbound forms for the further experiment because bound forms could have LPS toxin from *E. coli* when TRS was expressed. IL-1α is typical cytokine that have high biological activity as a positive control whereas IL-1β did not show biological activity in THP1 cells (**Figure 4**).

TRS leads biological activation in various type of cell line

Even though we confirmed human TRS have distinct biological activities in THP1 cell line, human TRS have same biological activities to other types of cell lines remain unknown, so after confirmed the biological activity of TRS in THP1 cells, I conducted the experiment with various cell lines with unbound forms of TRS with anion exchanged purification. HUVEC and A549 cells are reacted to TRS and induced IL-6 with dose dependently. Between two cells, HUVEC cells show higher production of IL-6 than A549 (**Figure 5A** and **5B**) However, it cannot induce biological activity with HMC1 cell line based on the IL-8 induction. (**Figure 5C**)

TRS induces IL-6 in primary cells

Human whole blood was drawn and separated into peripheral blood nonnuclear cells (PBMC) with ficoll-Paque^{TM} PLUS. TRS was used to treat whole blood cells and PBMC with various

concentrations (4, 20, 100 ng/mL). After incubation for 16 hours, the cell culture supernatants were harvested and measured for human IL-6 by using specific ELISA kit. Consequently, sufficient amount of IL-6 was produced in both cell culture supernatants of whole blood cells and PBMCs in a dose dependent manner. (Figure 6A and 6B)

TRS inducing cytokines in mouse cells lines

After human cells had been studied with recombinant human TRS, mouse cell lines were stimulated with human TRS to compare of cytokine activity of TRS between human and mice. Human TRS cytokine was treated with various concentrations of TRS in mouse cell lines. The next day, the cell culture medium was harvested, and murine TNFα concentrations were assessed using ELISA. As a result, murine TNFα was produced by a dose dependent manner. Mouse macrophage cell lines, J774a.1 (Figure 7A) and Raw 246.7 (Figure 7B), were used for studying cytokines-induced by TRS. Between two cells, Raw246.7 cells showed higher production of murine TNFα by stimulation of human TRS than that of J774a.1.

TRS stimulates inflammatory cytokines productions in primary mouse cells

Mice organs were separated and mashed to cell status. Cells of the organ were seeded in 96 well plated and treated with TRS. After 16 hours, supernatant were harvested and measured mouse IL-6 level by sandwich ELISA kit. Bone marrow derived cells, such as monocyte and macrophage cells showed the higher response in

IL-6 inductions with human TRS than other cell types. Murine IL-6 was induced in whole blood cells, Bone marrow cells and spleen (Figure 8A, B, and C). However, the same concentration of TRS failed to activate IL-6 level in lymph node (Figure 8D) and thymus (Figure 8E). This result suggested that type of myeloid cells were highly stimulated than other cell types.

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TRS stimulates inflammatory cytokines in mouse organs

Mouse primary cells showed the different response of murine IL-6 induction by human TRS depends on the type of cells. To characterize *in vivo* activity of TRS, human TRS injected to mice and checked the murine IL-6 concentration on tissue level by ELISA. Mice were pre-injected via intraperitoneal (IP) injection with TRS 10 μg per mouse. After 2 hours, mice were sacrificed, and organs were separated. Organs were homogenized, and total proteins were quantified with BCA. Mouse serum, bone marrow, and spleen produced IL-6 by TRS injection (Figure 9A, B, and C) while, lung shows small changes by TRS injection and higher murine IL-6 level in TRS non treated sample (control) than other cell types. (Figure 9D). The result shows similar tendency of murine IL-6 activation by human TRS with the type of mouse primary cell lines.

Full length TRS is required for cytokine activity

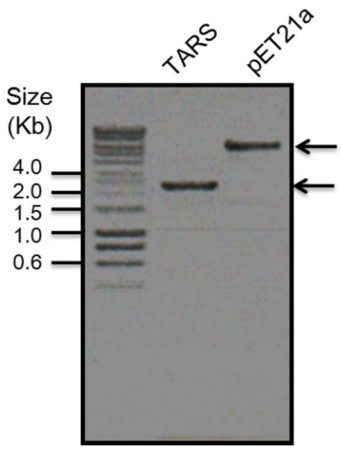
TRS fragments are designed to whether the enzyme activity of TRS could affect cytokine activity or not. There are two fragments (Figure 10A) of TRS which are N-terminal domain (UNE-T, 1-322 amino acid) the other is C-terminal domain

(Catalytic site and anticodon binding domain, 322-723 amino acid) which site has the catalytic activity of TRS. The experiment conducted with 100ng/ml of TRS proteins with the same way of as cytokine assay in method 4 with THP1 cells. Consequently, the fragments of TRS did not induce of IL-8 in THP1 cells. Only wild type of full lenth TRS showed the cytokine activity in THP1 cells (Figure 10B).

TRS stimulated signal pathways in THP1.

THP1 cells were treated with 100ng/ml of TRS for confirming which signal pathways are related to the TRS stimulation. Cells were treated with human TRS at different time points (0, 5, 15, 30, 60, 120 min), and harvested for western blotting. As a result, p44/42 and p38 proteins were phosphorylated after 5 min and phosphorylation levels were returned to original levels after 15 min (Figure 11A)

A



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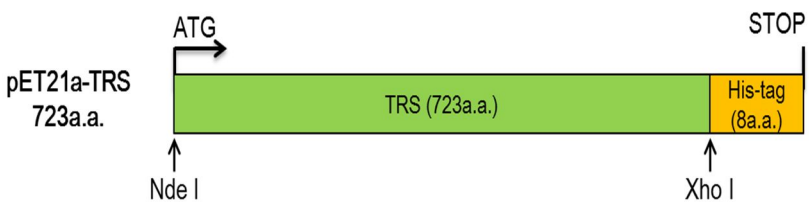


Figure 1. Threonyl- tRNA synthetase cloning

(A)TRS and pET21a vector were amplified by PCR and confirmed by agarose gel electrophoresis. (B) TRS was tagged with His^{6}-tag on the C-terminus.

A B

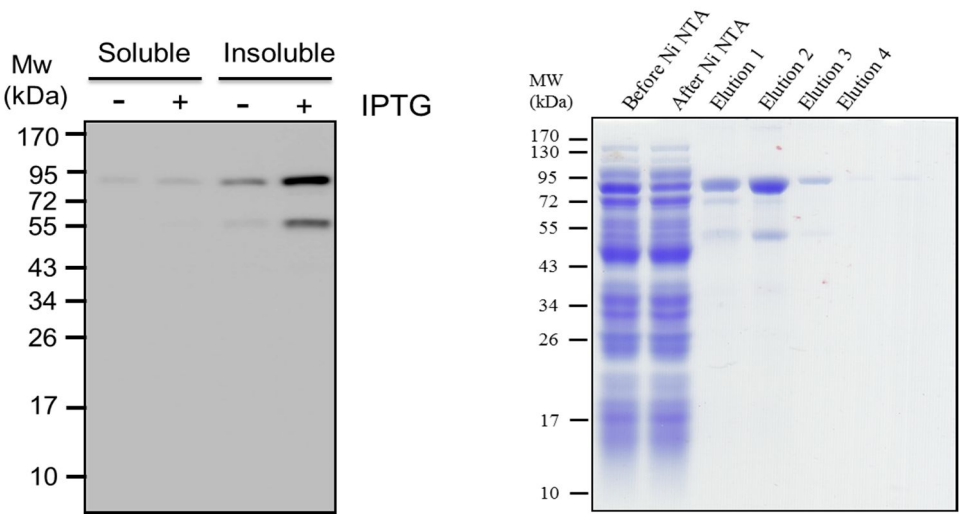
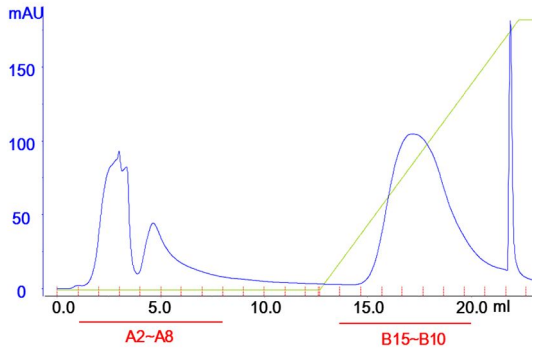


Figure 2. TRS expression and purification with Ni-NTA affinity column

Recombinant human TRS proteins were expressed in *E. coli* and purified by Ni-NTA affinity chromatography.(**A**)TRS expression were visualized by Western blot (1^{st} antibody, Mouse anti-his^{6} tag, 2^{nd} antibody, Goat anti-mouse HRP). (**B**) Eluted proteins were separated by 10% SDS-PAGE and stained by Coomassie blue staining.

A



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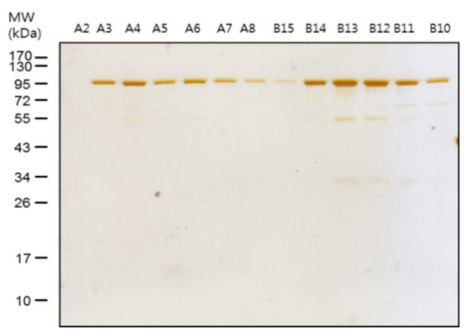

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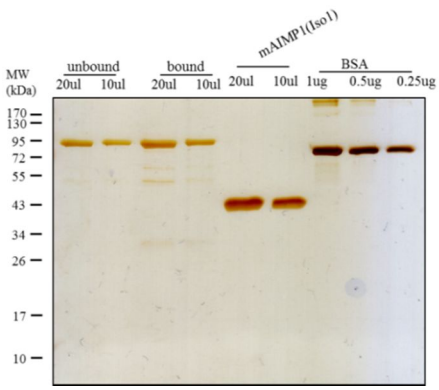


Figure 3. The second step purification of recombinant human TRS with anion exchange chromatography.

(A)Ni-NTA affinity column protein fractions are pooled and observed in FPLC peak (anion exchange). (B) FPLC purified TRS were analyzed by silver staining in 10% SDS-PAGE gel. (C)The purified TRS proteins were quantified with BSA (murine AIMP was loaded as control protein)

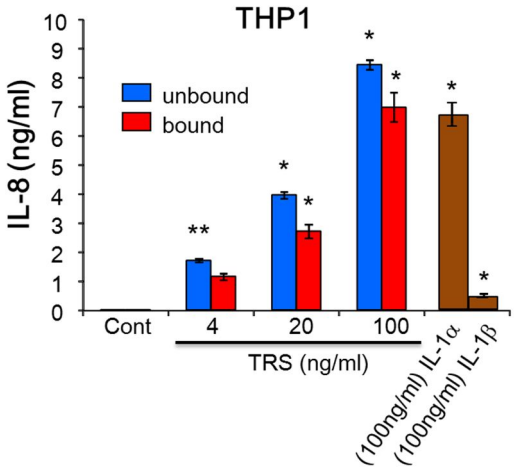


Figure 4. Biological activity of the two-step purified TRS

THP1 cells were seeded 5 x 10^4 per well in 96 well plates and purified human TRS was used to treat THP1 cells with various concentration (4, 20, 40 ng/mL with anion exchanged unbound and bound forms). After incubation for 16 hours, supernatants were harvested, and human IL-8 was measured by an IL-8 sandwich ELISA kit. Data are expressed as the Mean ± SEM; *, $p < 0.05$; **, $p < 0.01$ from duplicates.

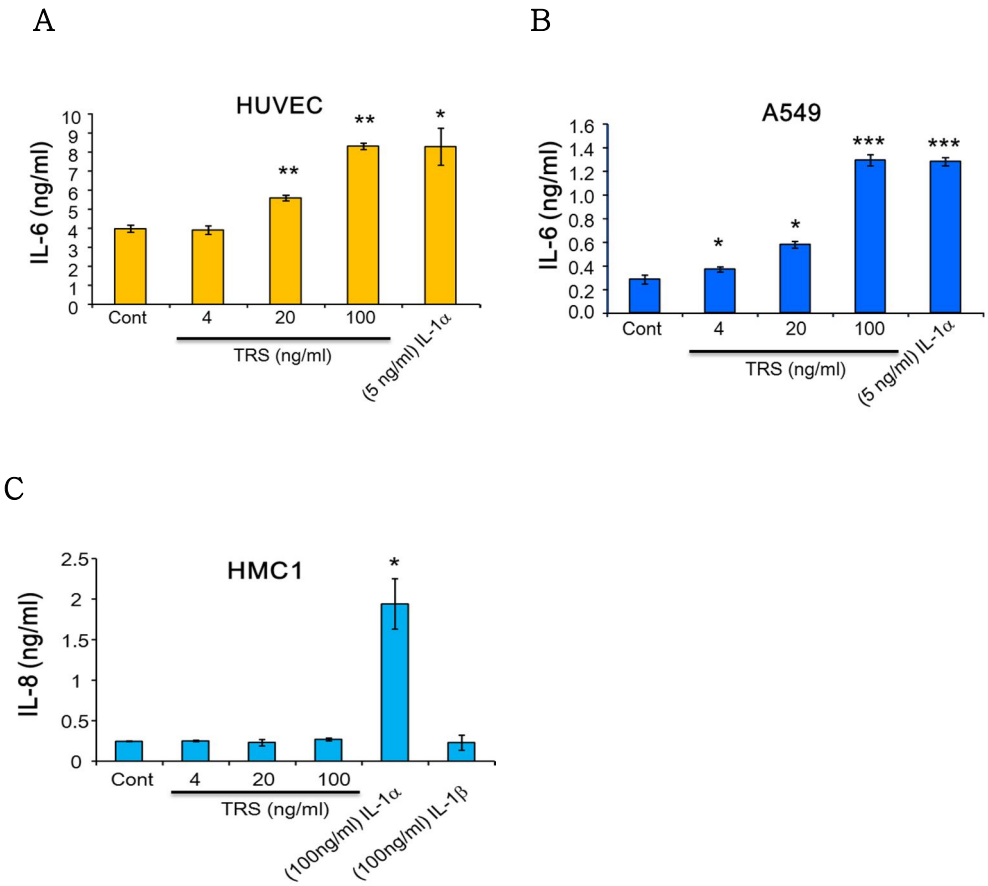


Figure 5. TRS mediates inflammatory cytokines production in a dose dependent manner in cell lines

Various cell lines (A) HUVEC, (B) A549, (C) HMC1 were treated with various concentrations of human TRS for confirming biological activity of TRS. After 16 hours, the supernatants were harvested and measured inflammatory cytokines such as human IL-6 and IL-8. Mean ± SEM; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (n=3).

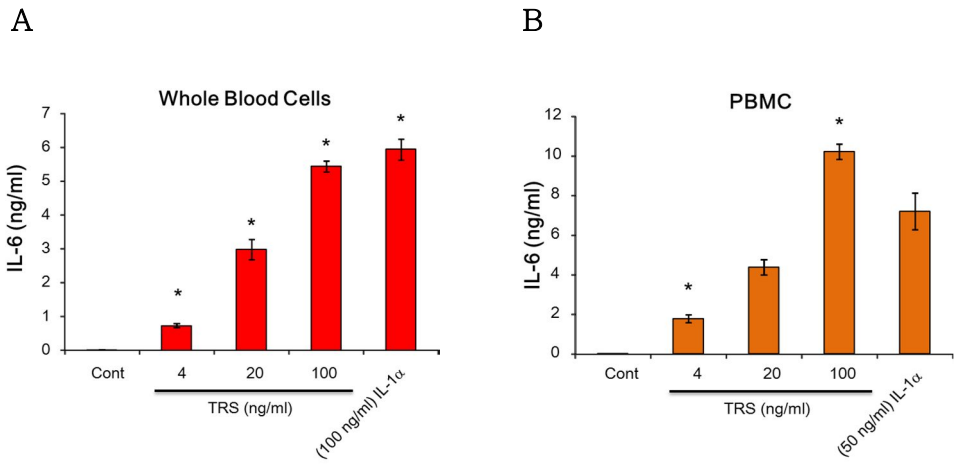


Figure 6. TRS induced IL-6 in primary cells

IL-6 production induced by TRS in Human whole blood cells (A), PBMC (B). The concentrations of TRS (4, 20, 100 ng/mL) and IL-1α are indicated at the bottom of the graph. Mean ± SEM; *, $p < 0.05$ (n=2).

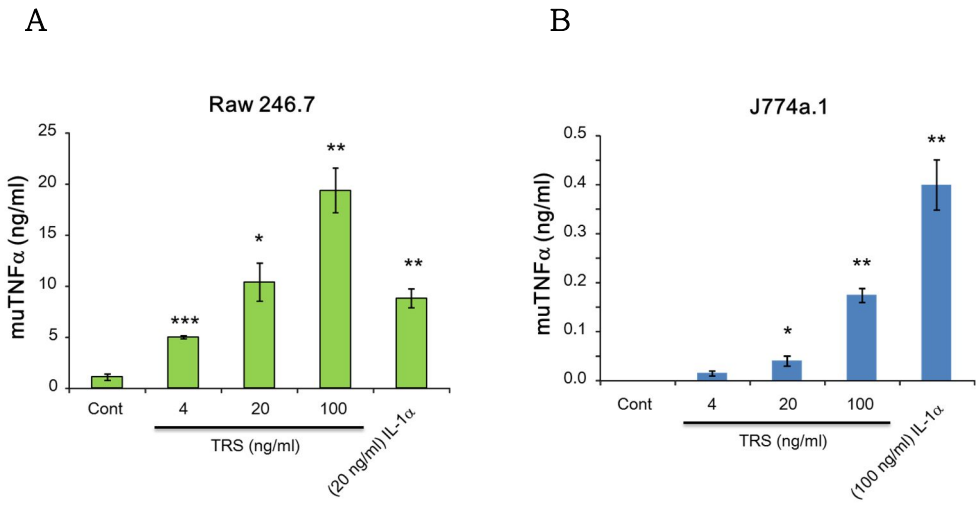


Figure 7. TRS mediated cytokines productions in mouse cells lines

Mouse TNFα was induced by TRS in mouse cells (Raw 246.7 (A), J774a.1 (B)). The concentration of TRS (4, 20, 100 ng/mL) and IL-1α are indicated at the bottom of the graph. Mean ± SEM; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (n=3).

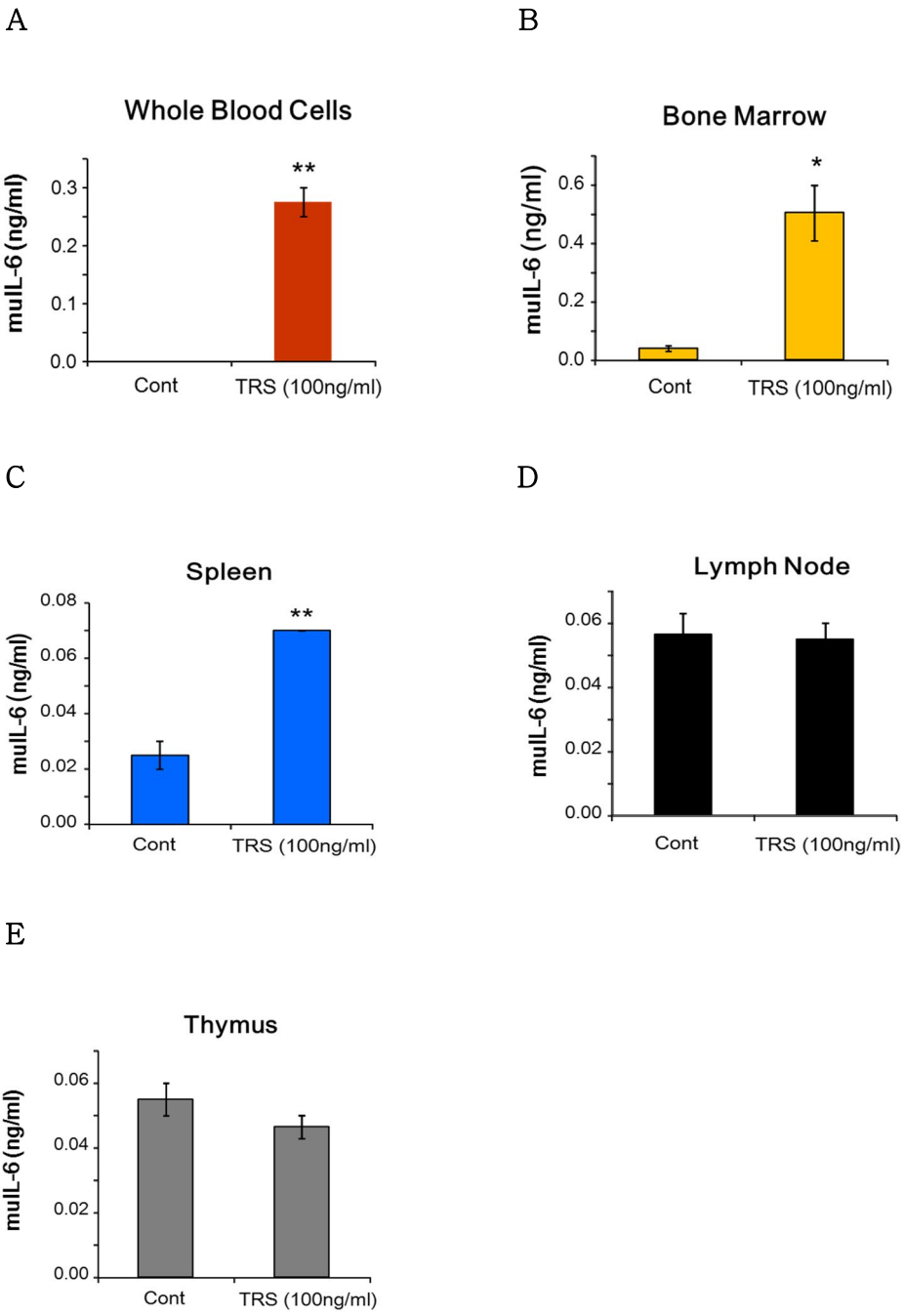


Figure 8. TRS stimulates inflammatory cytokines productions in primary mouse cells.

To prepare mouse primary cells, mice were dissected, and organs were separated (whole blood cells (**A**), bone marrow cells (**B**), spleen (**C**), lymph node (**D**), and thymus (**E**)). Organs were mashed and prepared as cell state. Cells were seeded 1 x 10^5 per well in 96 well plates. TRS was used to treat each well with 100 ng/ml. After 16 hours, supernatants were harvested and measured mouse IL-6 by ELISA kit. Mean ± SEM; *, $p < 0.05$; **, $p < 0.01$ (n=3).

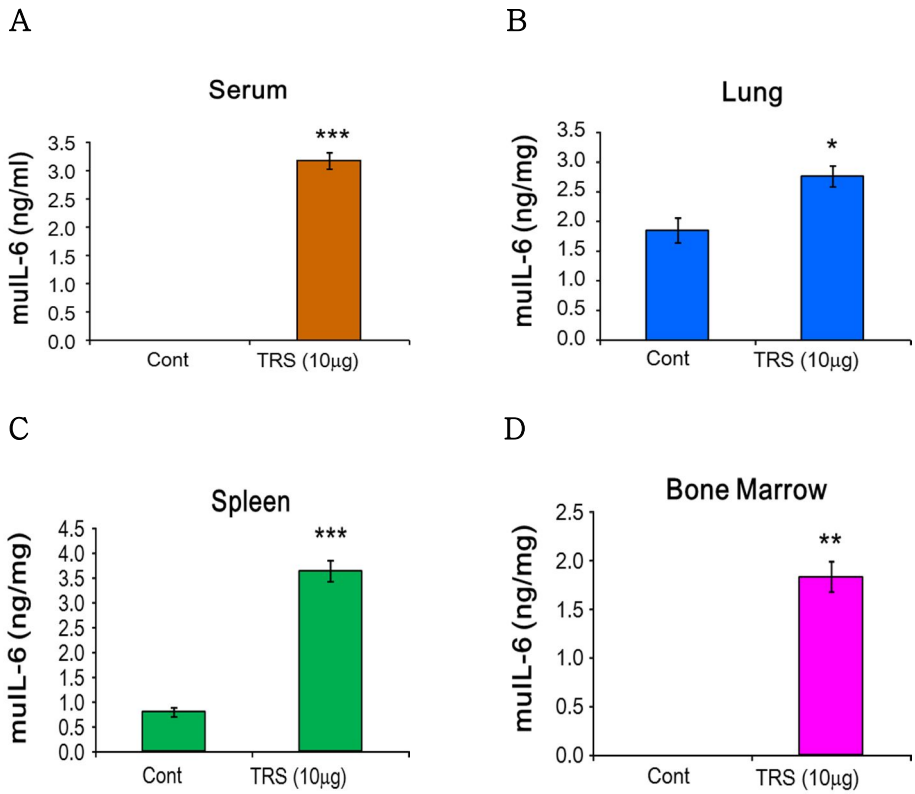
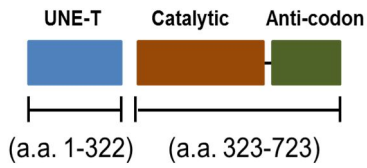


Figure 9. TRS induced murine IL-6 production in mouse tissues

TRS (10 μg) was injected via intra peritoneal (IP) injection to mice that were sacrificed after 2 hours. Tissues of organs were homogenized, and Murine IL-6 was measured by ELISA kit. Mean ± SEM; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (n=3).

A



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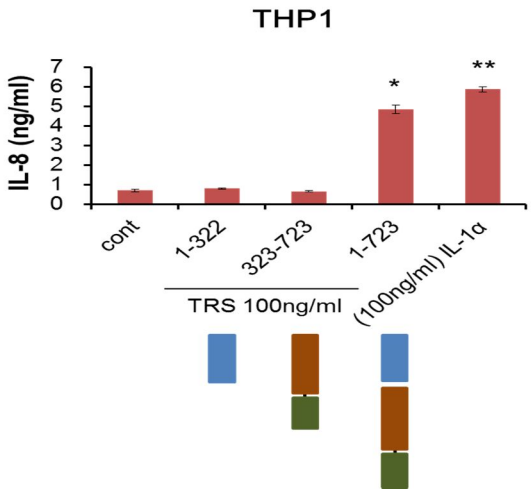

Figure 10. Full length TRS is required for cytokine activity

The schematic diagram of TRS (A), TRS was divided by N-terminal and C-terminal fragment. The only full length of TRS induced IL-8 on THP1 (B). Mean ± SEM; *, $p < 0.05$; **, $p < 0.01$ from duplicate.

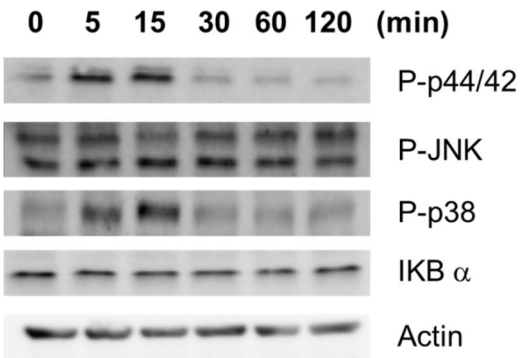

Figure 11. TRS stimulated signal pathways in THP1

THP was seeded 10^6/well in 6 well plates 2 hours before treatment of TRS with fetal bovine serum (FBS, Welgene) RPMI-1640 media. Cells were treated with human TRS (100ng/ml) at different time points (0, 5, 15, 30, 60, 120 min), and harvested for western blotting. Phosphorylated signaling proteins were detected with anti-phospho-protein antibodies.

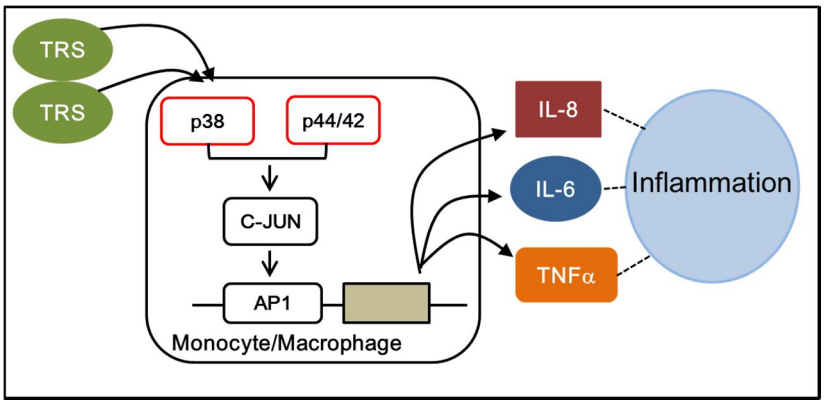

Figure 12. Schematic model of TRS signaling

Secreted TRS transfers the signals to other cells and stimulates inflammatory cytokines and amplifies immune responses.

DISCUSSION

ARSs are enzymes that tagging threonine to their cognate tRNA in protein synthesis. With protein synthesis regulators, ARS could be connected with various diseases (1). TRS is one of the ARS that transfers the threonine to its matched tRNA. Except the canonical function of TRS as enzyme activity, it is reported previously that TRS are secreted by other cytokines such as TNFα and VEGF and effects to other cells migration or angiogenesis (2). However, cytokines functions of TRS have remained to be explained.

At first, I expressed and purified recombinant TRS in *E.coli* for further studies. 2nd purification was conducted with anion exchange chromatography. With the two-step purified TRS, I confirmed the biological activity of TRS THP1 cell lines. Also, I confirmed cytokines activity of TRS by inducing IL-6, IL-8, and TNFα in various cell lines including *in vivo* studies. Inductions of those cytokines can be related to inflammatory immune responses or autoimmune diseases since the blockade of IL-6 and TNFα have been used in treatment of rheumatoid arthritis, inflammatory bowel disease and psoriasis. This concept is supported by studies that high TNFα level is a diagnostic marker in myositis that are related to anti-synthetase syndromes. The anti-TNFα therapy is most effective in myositis patients who have anti-TARS PL-7 antibodies (2, 12, 13). Also, other ARSs are concerned with immune cell reaction. For example, lysyl-, histidyl- and asparaginyl-tRNA synthetases enhance immune cell migration or cytokine release. (2, 5, 18)

In our study, it was proposed that secreted TRS can stimulate myeloid cells types, especially monocyte among various immune cells. Monocytes are part of the innate immune systems. So, TRS could be related to the innate immune response of monocytes. Also, the full length of TRS needed for the proinflammatory cytokine

function. It is recommended to identify the cell surface receptor of TRS for further study the specific mechanisms of TRS cytokine functions.

In this study, I suggested the novel function of TRS as the cytokine via activations of inflammatory signal pathways resulting in production of inflammatory cytokines. Also, TRS can be one of the potential markers for inflammatory autoimmune diseases.

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요약 (국문초록)

세포외로 분비된 Threonyl-tTRNA synthetase의 전염증성 기능 연구

김 해 준

의약생명과학전공
분자의학 및 바이오 제약 학과
융합과학기술대학원
서울대학교

ARS 는 아미노산을 각각의 짝이 되는 transfer RNA (tRNA)에 연결시켜주는 효소이다. 진핵 생물의 경우 20 가지의 ARS 가 존재하는데 TRS 는 Threonine 을 tRNA 에 전달 함으로써 단백질 합성을 조절한다. 하지만 이러한 전통적인 효소의 활성에 대해서는 많은 연구가 있었지만 그 외의 TRS 의 다른 역할에 대해서는 아직 알려진 바가 많지 않으며 여전히 연구되고 있다. 현재 외부의 VEGF, TNFα 등의 자극에 의해 TRS 는 세포 밖으로 분비되며 이 것은 는 암의 전이, 신 혈관 생성에 관여한다는 것이 알려져 있지만 아직까지 분비된 가 TRS 가 어떠한 역할을 하는지에 대해서는 모르고 있는 상태이다.
염증반응에 연관된 사이토카인은 세포 외 신호에 의해 활성화 되고 다시 세포 밖으로 분비되어 다른 세포에 신호를 전달함으로써 염증반응을 매개한다. 흥미롭게도 세포 밖으로 분비된 TRS 가 면역세포에서 중요한 싸이토 카인들을 활성화 시킨다는 것을 알게 되었고 이러한 결과를 통하여 TRS 사이토카인 으로써의 역할을 알아보기 위해 실험을 하였다. 본 연구에서는 외부로 분비된 TRS 가 면역반응에 관여하는 사이토카인 으로써의 역할을 확인하기 위해 재조합 단백질 기술을 이용하여 인간의 TRS 를 정제 하고 이를 이용하여 다양한 세포 주 에서 TRS 에 대한 반응을 ELISA 실험을 통해 확인 하였으며 결과적으로 TRS 에 의해 염증 반응에 관여 하는 사이토카인들을 활성화 시킨다는 것을 확인하였다. 이러한 결과로 TRS 는 세포 사이의 여러 가지 정보전달

에 관여하는 단백질이며 새로운 면역반응의 마커와 염증성 질환 연구의 새로운 타겟으로 써의 역할을 할 수 있을 것이라 예상된다.

주요어 : 트레오닌-트랜스퍼 RNA 합성효소, 염증성 싸이토카인

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