# 学 位 論 文 の 要 旨

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学 位 論 文 名　TGF-β1 Enhances Degradation of IFN-γ-induced iNOS Protein via Proteasomes in RAW 264.7 Cells

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## 論 文 内 容 の 要 旨

### INTRODUCTION

Macrophages are activated by interferon-γ (IFN-γ), released from activated $CD4^+$ or $CD8^+$ T cells and natural killer cells, to induce the expression of several genes such as inducible nitric oxide synthase (iNOS). Nitric oxide (NO) is produced by iNOS and plays important roles in immune responses and pathogenesis of some diseases. However, iNOS-derived NO concentration must be kept within appropriate range, since the excessive amounts of NO could cause harmful conditions including progressions of septic shock and tissue injuries. Transforming growth factor-β1 (TGF-β1), a well-known immunosuppressive factor, was shown to suppress iNOS gene expression at transcriptional and posttranscriptional levels. It has been reported that iNOS protein level is regulated by ubiquitin-proteasome pathway, however it remains unclear whether TGF-β1 controls ubiquitin-proteasome pathway. In this study, we demonstrate that TGF-β1 enhances degradation of IFN-γ-induced iNOS protein via ubiquitin-proteasome pathway in murine macrophage-like cell line RAW 264.7.

## MATERIALS AND METHODS

RAW 264.7 cells obtained from Riken Cell Bank were used for this study. Nitrite in culture medium was measured by fluorometric assay using 2,3-diaminonaphthalene. Quantifications of iNOS mRNA and iNOS protein were performed by real-time quantitative PCR and Western blot analysis using anti-iNOS antibody, respectively. To estimate iNOS protein degradation *in vitro*, lipopolysaccharide plus IFN-γ-treated cell lysate (as substrate) and IFN-γ- or IFN-γ plus TGF-β1-treated cell lysate depleted of iNOS protein using anti-iNOS antibody (as enzyme) were incubated with ATP regeneration system containing 4 mM ATP, 0.2 U creatine kinase and 10 mM creatine phosphate. Ubiquitinated iNOS protein was detected by immunoprecipitation and Western blot analysis using anti-iNOS and anti-ubiquitin antibodies, respectively. Trypsin-like (T-L) activity was determined by measuring fluorescent intensity of fluorogenic peptide substrate (*t*-butyloxycarbonyl-L-leucyl-L-arginyl-L-arginine 4-methylcoumaryl-7-amide).

## RESULTS AND DISCUSSION

Addition of 0.1, 1 and 10 ng/ml TGF-β1 into the culture medium of RAW 264.7 cells dose-dependently reduced nitrite accumulation induced by 100 U/ml IFN-γ. Thereafter, the effects of TGF-β1 on mRNA and protein levels of iNOS were analyzed. TGF-β1 treatment did not affect IFN-γ-induced iNOS mRNA level, whereas IFN-γ-induced iNOS protein level was decreased by TGF-β1 treatment. In addition, the decreased iNOS protein level was not detected in the presence of MG132, a proteasome inhibitor, indicating that TGF-β1 treatment does not affect IFN-γ-induced iNOS protein synthesis. To investigate whether TGF-β1 is involved in ubiquitin-proteasome pathway, which degrades

proteins in an ATP-dependent manner, iNOS protein degradation assay *in vitro* was carried out. IFN-γ plus TGF-β1 treatment enhanced the degradation of iNOS protein compared with IFN-γ treatment alone. In addition, the enhanced iNOS protein degradation was blocked by MG132, and never observed without ATP regeneration system. These results suggest that TGF-β1 might enhance iNOS protein degradation via ubiquitin-proteasome pathway. It was examined whether TGF-β1 facilitates iNOS protein ubiquitination. Immunoprecipitation assay revealed that IFN-γ plus TGF-β1 treatment did not affect the level of ubiquitinated iNOS protein compared with IFN-γ treatment alone. Then, three major proteasomal proteolytic activities in mammalian cells, such as peptidylglutamyl-peptide hydrolyzing, chymotrypsin-like and T-L activities, were measured. Among the tested proteolytic activities, T-L activity was prominently enhanced by IFN-γ plus TGF-β1 treatment compared with IFN-γ treatment alone. Furthermore, leupeptin, an inhibitor of T-L protease, blocked the degradation of iNOS protein *in vitro*, indicating that the enhanced T-L activity of protease is involved in iNOS protein degradation. Taken together, these results strongly indicate that TGF-β1 enhances iNOS protein degradation via ubiquitin-proteasome pathway by increasing T-L activity of proteasomes, and thus decreases iNOS protein level.

## CONCLUSION

TGF-β1 enhances degradation of IFN-γ-induced iNOS protein via ubiquitin-proteasome pathway and decreases iNOS protein and NO production by increasing T-L activity of proteasomes rather than promoting ubiquitination of iNOS protein.