学 位 論 文 の 要 旨

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学　位　論　文　名	Ubiquitin-like Protein MNSFβ Covalently Binds to Bcl-G and Enhances Lipopolysaccharide (LPS)/Interferon γ (IFNγ)-induced Apoptosis in Macrophages
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論 文 内 容 の 要 旨

INTRODUCTION

In eukaryotes, a major mechanism for regulating protein activity involves covalent attachment of ubiquitin or ubiquitin-like proteins to the ε-amino group of lysine via the formation of an isopeptide bond. Post-translational modification by ubiquitin-like proteins regulates a variety of important eukaryotic processes, such as cell division, nuclear transport, the stress response, and immune responses. Monoclonal non-specific suppressor factor β (MNSFβ) was originally found as a cytokine. MNSFβ presents a 57% homology with ubiquitin. The C-terminal amino acids of ubiquitin (Gly-Gly), involved in the isopeptide bond formation during conjugation, are conserved in the MNSFβ sequence. Unlike ubiquitin, MNSFβ might not be involved in protein degradation. MNSFβ is covalently attached to certain lysines of various intracellular target proteins, including Bcl-G, a pro-apoptotic member of the Bcl2 family, and endophilin II. MNSFβ conjugates to Bcl-G with a linkage between the C-terminal Gly74 and Lys110 and regulates the mitogen-activated protein kinase (MAP kinase) pathway by inhibiting the activation of extracellular signal-regulated kinase (ERK). In this study, we demonstrate that MNSFβ covalently conjugates to Bcl-G and promotes LPS/IFNγ-induced apoptosis in mouse macrophage-like cell line Raw264.7.

MATERIALS AND METHODS

Raw264.7 cells were treated with siRNAs directed against MNSFβ and Bcl-G for 48 h in

the presence of the HiPerFect Transfection Reagent. cDNAs encoding MNSFβ and Bcl-G were subcloned into the vector pcDNA3.1(+). Transient DNA transfections were conducted using Lipofectamine Plus reagent and plasmid DNA. Mutant Bcl-G (K110R) was generated by replacing the codon for lysisne 110 with the codon for arginine. Apoptotic cells were stained using APOPercentage™ apoptosis assay. Stained cells were analysed on NHI ImageJ software and Adobe Photoshop. Nitric oxide (NO) production in culture medium was determined by using the Griess reagent. For Western blot analysis, equal amounts of protein (whole cell lysates) were loaded onto an SDS-polyacrylamide gel, resolved by electrophoresis, and transferred onto polyacrylamide vinylidene fluoride membranes. The membranes were incubated first with primary antibodies and then with secondary antibodies conjugated with horseradish peroxidase, washed, and developed with the enhanced chemiluminescence kit. For electrophoretic mobility shift assay (EMSA), protein-DNA complexes were detected using biotin end-labeled double-stranded DNA probes. The binding reaction was performed using the EMSA "Gel Shift" Kit. Peritoneal macrophages were obtained using BALB/c mice injected with 4% brewer thioglycolate broth. The collected macrophages were used for apoptosis studies. All experiments were approved and conducted in accordance with the guidelines of the Animal Care Committee of the Shimane University.

RESULTS AND DISCUSSION

In Raw264.7 cells treated with MNSFβ siRNA LPS/IFNγ-induced apoptosis was significantly inhibited. Conversely, transfection with pcDNA3.1–MNSFβ resulted in the enhancement of apoptosis induced by LPS/IFNγ, indicating that this ubiquitin-like protein may mediate pro-apoptotic signal transduction. Transfection with pcDNA3.1–Bcl-G resulted in a small enhancement of LPS/IFNγ-induced apoptosis. However, co-transfection of the expression vectors encoding Bcl-G and MNSFβ caused a marked enhancement of apoptosis (83%±5). These results strongly suggest that MNSFβ•Bcl-G complex may play an essential role in the positive regulation of apoptosis in macrophages. Supporting this notion, transfection of mutant Bcl-G (K110R) construct exerted no effect on pro-apoptotic function. Collectively, MNSFβ conjugation to Bcl-G with a linkage between the C-terminal Gly74 and Lys110 is responsible for the regulation of apoptosis. It has been reported that LPS/IFNγ-induced apoptosis in Raw264.7 cells is mediated by NO. Western blot analysis showed that iNOS expression was not affected by the treatment with MNSFβ siRNA. In addition, siRNAs directed against MNSFβ and Bcl-G did not affect NO production in LPS/IFNγ-activated cells. In contrast, NO-induced apoptosis was significantly inhibited in the cells transfected with MNSFβ siRNA, indicating that MNSFβ•Bcl-G complex may be involved in the signal pathway downstream of NO production in LPS/IFNγ-stimulated macrophages.

Special emphasis was placed on p53, because *BCL-G* gene is a pro-apoptotic p53 target gene. It is also evident that NO induces p53 accumulation in Raw264.7 cells. Double knockdown of Bcl-G and MNSFβ resulted in a marked decrease in the amount of p53 in LPS/IFNγ-stimulated cells. Conversely, the expression of p53 was strongly increased in the cells co-transfected with MNSFβ and Bcl-G. We also observed inhibition of both poly (ADP-ribose) polymerase cleavage and Caspase-3 activation by the double knockdown of Bcl-G and MNSFβ. These results indicate that MNSFβ•Bcl-G complex may act upstream of p53 and positively regulate the level of p53. It has been reported that the induction of Cox-2 represents an essential regulator of NO-mediated apoptosis in Raw264.7 cells. Co-overexpression of MNSFβ and Bcl-G led to strong inhibition of the Cox-2 expression induced by LPS/IFNγ. We did not observe significant changes in the level of Cox-2 expression in mutant MNSFβ (G74A) cDNA transfected cells. The Cox-2 expression induced by S-nitrosoglutathione (GSNO) was also inhibited, demonstrating that MNSFβ•Bcl-G promotes apoptosis, in part, through inhibiting Cox-2 expression. Our observations further suggest that Cox-2 expression and p53 accumulation are inversely related in RAW 264.7 macrophages.

LPS/IFNγ-induced macrophage activation leads to a variety of signal transduction pathways, including all three MAPK cascades, i.e., ERK, p38 and JNK pathways. We have previously demonstrated that MNSFβ covalently binds to Bcl-G and regulates ERK-MAPK cascade. In Raw264.7 cells co-transfected with MNSFβ and Bcl-G cDNAs, LPS-induced ERK1/2 phosphorylation was strongly inhibited. This co-overexpression also inhibited GSNO-induced ERK1/2 phosphorylation. This observation suggests that MNSFβ•Bcl-G is involved in both early and late phase ERK1/2 phosphorylation leading to Cox-2 activation. NFκB and AP-1 are involved in the LPS signaling cascade leading to Cox-2 production. EMSA demonstrated that double knockdown of MNSFβ and Bcl-G strongly enhanced AP-1 activation. We have previously reported that MNSFβ slightly affects LPS-induced NFκB signaling. Thus, it is conceivable that MNSFβ affects ERK/AP-1 signaling rather than NFκB signaling.

In LPS/IFNγ-stimulated peritoneal macrophages, co-overexpression of MNSFβ and Bcl-G caused a marked enhancement of apoptosis. Furthermore, Western blot analysis showed that Cox-2 expression was strongly inhibited by this co-overexpression. Therefore, our observation that MNSFβ•Bcl-G enhances LPS/IFNγ-induced apoptosis in RAW264.7 cells is confirmed in primary cultured peritoneal macrophages.

CONCLUSION

The present study demonstrates that MNSFβ•Bcl-G formation might be important for potent regulation of apoptosis in macrophages and that the apoptosis enhancing effect of this complex is due in part to the down-regulation of Cox-2 activation.