

18. Miwa K, Kishimoto C, Nakamura H, Makita T, Ishii K, Okuda N, et al. Serum thioredoxin and α -tocopherol concentrations in patients with major risk factors. *Circ J* 2005; **69**: 291–294.
19. Eaton P, Bell RM, Cave AC, Shattock MJ. Ischemic preconditioning: A potential role for protein S-thiolation? *Antioxid Redox Signal* 2005; **7–8**: 882–888.
20. Yokoi H, Nobuyoshi M, Mitsudo K, Kawaguchi A, Yamamoto A, ATHEROMA Study Investigators. Three-year follow-up results of angiographic intervention trial using an HMG-CoA reductase inhibitor to evaluate retardation of obstructive multiple atheroma (ATHEROMA) Study. *Circ J* 2005; **69**: 875–883.
21. Maejima Y, Ysu T, Ueba H, Kobayashi N, Hashimoto S, Kubo N, et al. Exercise after heparin administration: New therapeutic program for patients with non-option arteriosclerosis obliterans. *Circ J* 2005; **69**: 1099–1104.
22. Cha MK, Kim IH. Disulfide between Cys392 and Cys438 of human serum albumin is redox-active, which is responsible for the thiorexin-supported lipid peroxidase activity. *Arch Biochem Biophys* 2006; **445**: 19–25.
23. Jung CH, Thomas JA. S-glutathionylated hepatocyte proteins and insulin disulfides as substrates for reduction by glutaredoxin, thioredoxin, protein disulfide isomerase, and glutathione. *Arch Biochem Biophys* 1996; **335**: 61–72.
24. Mukai E, Kume N, Hayashida K, Minami M, Yamada Y, Seino Y, et al. Heparin-binding EGF-like growth factor induces expression of lectin-like oxidized LDL-receptor-1 in vascular smooth muscle cells. *Atherosclerosis* 2004; **176**: 289–296.
25. Barros MR, Bertolami MC, Aballa DS, Ferreira EP. Identification of mildly oxidized low-density lipoprotein (electronegative LDL) and its auto-antibodies IgG in children and adolescents hypercholesterolemic offspring. *Atherosclerosis* 2006; **184**: 103–107.
26. Zhang B, Noda K, Matsunaga A, Kumagai K, Saku K. A comparative crossover study of the effects of fluvastatin and pravastatin (EP-COS) on circulating autoantibodies to oxidized LDL in patients with hypercholesterolemia. *J Atheroscler Thromb* 2005; **12**: 41–47.
27. Yasue H, Hirai N, Mizuno Y, Harada E, Itoh T, Yoshimura M, et al. Low-grade inflammation, thrombogenicity, and atherogenic lipid profile in cigarette smokers. *Circ J* 2006; **70**: 8–13.
28. Nagai K, Betsuyaku T, Kondo T, Nasuhara Y, Nishimura M. Long term smoking with age builds up excessive oxidative stress in bronchoalveolar lavage fluid. *Thorax* 2006; **61**: 496–502.



Alpha-defensin enhances expression of HSP47 and collagen-1 in human lung fibroblasts

Sumako Yoshioka^a, Hiroshi Mukae^{a,*}, Hiroshi Ishii^a, Tomoyuki Kakugawa^a, Hiroshi Ishimoto^a, Noriho Sakamoto^a, Takeshi Fujii^a, Yoshie Urata^b, Takahito Kondo^b, Hiroshi Kubota^c, Kazuhiro Nagata^c, Shigeru Kohno^a

^a Second Department of Internal Medicine, Nagasaki University School of Medicine, 1-7-1 Sakamoto, Nagasaki, 852-8501 Japan

^b Department of Biochemistry and Molecular Biology in Disease, Nagasaki University School of Medicine, Nagasaki, Japan

^c Department of Molecular and Cellular Biology, Institute for Frontier Medical Science, Kyoto University, Kyoto, Japan

Received 4 September 2006; accepted 14 February 2007

Abstract

Neutrophils and lung fibroblasts are thought to play a role in the pathogenesis of pulmonary fibrosis. We reported previously that heat shock protein 47 (HSP47), a collagen-specific molecular chaperon, and collagen-1 synthesis were involved in pulmonary fibrosis, and that plasma levels of α -defensins (HNP; human neutrophil peptide), cationic proteins with antimicrobial and cytotoxic activity in neutrophils, were significantly higher in patients with idiopathic pulmonary fibrosis than in control subjects. Here, we investigated the direct effect of HNP-1 *in vitro* on the expression of HSP47 and collagen-1 in human lung fibroblasts (NHLF). HNP-1 at 5 μ g/ml induced fibroblast proliferation but at concentrations >50 μ g/ml, HNP-1 reduced cell viability. Incubation of NHLF with 10 to 25 μ g/ml of HNP-1 for 24-h increased the expression of HSP47 and collagen-1 mRNAs ($p < 0.05$). The levels of HSP47 protein also increased significantly at 50 μ g/ml, and those of collagen-1 protein increased at 10 to 50 μ g/ml of HNP-1 ($p < 0.05$). The mitogen-activated protein kinase (MAPK) signaling pathway in NHLF was activated by HNP-1 stimulation, but inhibitor of MEK (PD98059) did not block HNP-1-induced HSP47 protein production. Our results suggest that α -defensin is a fibrogenic mediator that promotes collagen synthesis through the upregulation of HSP47 and collagen-1 in lung fibroblasts and participates in the pathogenesis of neutrophil-induced pulmonary fibrosis.

© 2007 Elsevier Inc. All rights reserved.

Keywords: Human neutrophil peptide-1; HSP47; Collagen synthesis; Pulmonary fibrosis

Introduction

Neutrophils are considered to play a pathologic role in acute and chronic injurious diseases of the lung. A marginal increase of neutrophils in the respiratory tract can be associated with major damage and irreversible architectural changes in the lung (Sibille and Marchandise, 1993). Idiopathic pulmonary fibrosis (IPF) is a progressive and usually fatal lung disease characterized by patchy fibrotic areas with fibroblast proliferation and extracellular matrix remodeling, which result in irreversible distortion of the lung architecture. Increasing scientific evidence defines the importance of neutrophils in the pathogenesis of

pulmonary fibrosis (Crystal et al., 1984; Hunninghake et al., 1981; Obayashi et al., 1997; Turner-Warwick and Haslam, 1987; Wells et al., 1998). For example, large numbers of neutrophils were found in the bronchoalveolar lavage (BAL) fluid (Crystal et al., 1984; Hunninghake et al., 1981) and lung tissue of patients with IPF (Hunninghake et al., 1981; Obayashi et al., 1997). Furthermore, BAL neutrophil counts in IPF patients correlated with the severity of the disease as determined by chest CT (Wells et al., 1998), poor treatment outcome (Turner-Warwick and Haslam, 1987) and disease activity (Crystal et al., 1984). However, other studies reported that the number and proportion of neutrophils in BAL fluid did not correlate with the activity of neutrophil alveolitis and that they had limited prognostic value (Boomars et al., 1995; Schwartz et al., 1994). Thus, the exact role of neutrophils in the pathogenesis of IPF remains unclear.

* Corresponding author. Tel.: +81 95 849 7273.

E-mail address: hmukae@net.nagasaki-u.ac.jp (H. Mukae).

α -Defensins are cationic proteins with antimicrobial activity, which constitute 5–7% of the total protein content of the human neutrophil and 30–50% of the total protein content of the azurophilic granules (van Wetering et al., 1999). They are also one of the products of activated and deteriorating neutrophils and released extracellularly during inflammatory reactions (Ashitani et al., 1998a,b). Six α -defensin peptides have so far been isolated and characterized in human. Since they are mainly present in neutrophil azurophilic granules, human α -defensin-1 to -4 are conventionally called human neutrophil peptides (HNP)-1 to -4 (van Wetering et al., 1999). High concentrations of HNP-1, -2 and -3 were observed in various inflammatory and fibrotic lung diseases including IPF (Ashitani et al., 1998a,b, 2004; Mukae et al., 2002), suggesting that α -defensins are related to the pathogenesis of neutrophil-related lung diseases. In these reports, the mean plasma levels of HNP-1 to 3 in IPF (768.2 ± 422.4 ng/ml, \pm SD) and acute respiratory distress syndrome (2010 ± 1483 ng/ml) were significantly higher than in control subjects (200 to 300 ng/ml). In IPF, we have shown previously that the concentrations of HNP-1 to 3 in plasma correlated inversely with respiratory function tests and they reflected the clinical course with an acute exacerbation (Mukae et al., 2002). Immunohistochemically, positive staining for α -defensins was observed inside and outside neutrophils in the alveolar septa, especially in dense fibrotic areas (Mukae et al., 2002). These results suggest that neutrophils could be involved in the lung fibrotic process via the expression of α -defensins.

Heat shock protein 47 (HSP47), localized in the endoplasmic reticulum, is involved in the synthesis/assembly of various collagens as a collagen-specific molecular chaperone (Matsuoka et al., 2004; Nagai et al., 2000; Nakai et al., 1992). Expression of HSP47 has been reported to increase in parallel with the expression of collagens during the progression of various fibrosis models (Masuda et al., 1994; Pan and Halper, 2003). Indeed, strong immunostaining of both HSP47 and procollagen-I was noted predominantly in interstitial spindle-shaped cells around active fibrotic areas in the lung of patients with IPF and murine bleomycin-induced pulmonary fibrosis (Ishii et al., 2003; Kakugawa et al., 2004, 2005). These findings suggest that lung fibroblasts and myofibroblasts play a role in fibrotic process through the upregulation of HSP47.

In this study, to assess the relationship between α -defensin and lung fibrotic process, we examined the expression of HSP47 and collagen-1 in primary cultures of human lung fibroblasts following stimulation with HNP-1 or transforming growth factor (TGF)- β 1, which is a well-known chemotactic factor for fibroblasts and promotes the production of extracellular matrix proteins such as collagen.

Materials and methods

Cells and agents

Normal human lung fibroblasts (NHLF) obtained from three different donors were purchased from Clonetics-BioWhittaker (Walkersville, MD). Cells were grown in fibroblast basal medium (FBM; Clonetics-BioWhittaker) supplemented with 2% fetal

bovine serum, human recombinant fibroblast growth factor (1.0 μ g/ml), insulin (5 mg/ml), gentamicin and amphotericin-B in humidified incubator (5% CO₂ at 37 °C). The synthetic products of HNP-1 and TGF- β 1 were purchased from Peptide Institute (Osaka, Japan) and R&D Systems (Minneapolis, MN), respectively.

Subculture and stimulation of NHLF

For experiments to evaluate the mRNA and protein expression levels of HSP47 and collagen-1, subcultures of NHLF were plated in 60-mm dishes (BD Falcon™, Franklin Lakes, NJ) at a density of 5×10^5 cells/dish. When the cells reached a confluence of approximately 70–80%, the medium was replaced by serum-free FBM. Cells were subsequently stimulated for 3, 6, 9, 12, 24 and 36 h with medium alone (control), synthetic HNP-1 5 to 100 μ g/ml or TGF- β 1 1 to 10 ng/ml. To examine the activation of the mitogen-activated protein kinase (MAPK) signaling pathway, NHLF were plated in 6-well dishes at a density of 5×10^4 cells per well and cultured to 70–80% confluence. After overnight starvation in serum-free FBM, the cells were incubated with 10 μ g/ml HNP-1 in serum-free medium for 15 min to detect the phosphorylated ERK-1/2, or 24 h to confirm the production of HSP47 protein. In addition, the cells were incubated with PD98059 (50 μ M; Alexis, Nottingham, UK) 1 h before addition of HNP-1.

Alamar Blue reduction assay

After 24-h incubation with HNP-1, the cells were treated for 3 h with 10-fold diluted Alamar Blue (Serotec, Oxford, UK) with FBM. Cell viability, the reducing environment of proliferating cells, was measured at 540 nm excitation and 620 nm emission. The results were compared with non-treated control cells.

Northern blot analysis

Human HSP47 and collagen-1 complementary DNAs (cDNAs) were generously provided by the Institute for Frontier Medical Science, Kyoto University, Japan. HSP47 mRNA was detected by using a 1.5 kilobase pair (kbp) *EcoR*-I fragment of full-length human HSP47 (CBP2) cDNA probe, whereas collagen-1 mRNA was detected by using a 0.6 kbp *EcoR*-V of full-length pCOLA1-I-CP cDNA probe. Total RNA was extracted from NHLF using RNeasy Mini Kit (Qiagen, Tokyo) using the method recommended by the supplier. Aliquots of total RNA (10 μ g/lane) were size-fractionated in a 1.2% formaldehyde agarose gel and transferred to a positively charged nylon membrane (Amersham Biosciences Corp., Piscataway, NJ). The membrane was separately hybridized with ³²P-labeled HSP47 and collagen-1 cDNAs for 20 h at 42 °C. Autoradiographed membranes were analyzed using a BAS5000 bioimage analyzer (Fuji Photo Film). Relative transcription was normalized with GAPDH probe hybridization.

Western blotting

Cells were washed with phosphate-buffered saline (PBS) and solubilized in lysis buffer (20 mM Tris-HCl, 133 mM NaCl, 1%

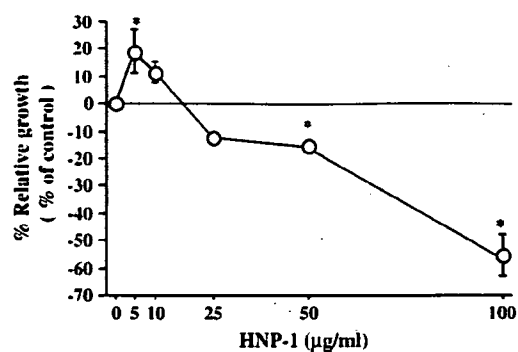


Fig. 1. Influence of HNP-1 on proliferation of normal human lung fibroblasts (NHLF) measured by Alamar Blue reduction assay. NHLF were separately incubated for 24 h with 0 (control) to 100 µg/ml of HNP-1. The growth levels were compared with non-treated control cells and the resulting ratio is shown as the percentage change from control values. Data are mean±SEM of four experiments. * $p < 0.05$, compared with the control.

NP-40, 10% glycerol, 0.2 mM PMSF, 1 µg/ml pepstatin and leupeptin). The lysates were centrifuged at 15,000 ×g for 10 min at 4 °C and the supernatant was used as the cytoplasmic extract. Each extract was subjected to SDS-PAGE using 10% polyacrylamide gels. After electrophoresis the fractionated protein was transferred to a nitrocellulose transfer membrane (Pall Corporation, East Hills, NY). The membrane was blocked for 60 min at room temperature in 5% non-fat milk in Tris-buffered saline (pH

7.5) containing 0.05% Tween 20, and then incubated overnight at 4 °C in 5% non-fat milk in Tris-buffered saline (pH 7.5) containing 0.05% Tween 20 with 1 µg/ml the mouse monoclonal anti-HSP47 antibody (StressGen Biotechnologies, Victoria, BC, Canada), 1 µg/ml mouse monoclonal anti-ERK1/2 antibody (Cell Signaling, Beverly, MA) or 1 µg/ml mouse monoclonal anti-GAPDH antibody (Santa Cruz Biotechnology, Inc, Santa Cruz, CA). Excess antibody was washed away with Tris-buffered saline (pH 7.5) containing 0.01% Tween 20. The nitrocellulose membranes were incubated for 30 min at room temperature in Tris-buffered saline (pH 7.5) containing 0.05% Tween 20 and the secondary antibody (1:2000 dilution in Tris-buffered saline, pH 7.5, containing 0.05% Tween 20) (Peroxidase-conjugated rabbit anti-mouse immunoglobulins) (DAKO, Kyoto, Japan) followed by washing in Tris-buffered saline (pH 7.5) containing 0.01% Tween 20. The detection was performed using the ECL Plus Western Blotting Detection Reagents kit (Amersham Biosciences Corp). The intensity of HSP47 was correlated using mouse anti-actin monoclonal antibody (Chemicon International, Temecula, CA) calculated by densitometry.

Enzyme-linked immunosorbent assay (ELISA)

Cell culture supernatants were collected 24 h after addition of HNP-1 or TGF-β1 suspension, centrifuged and stored at -80 °C. Collagen-1 C-terminal telopeptide levels were measured using

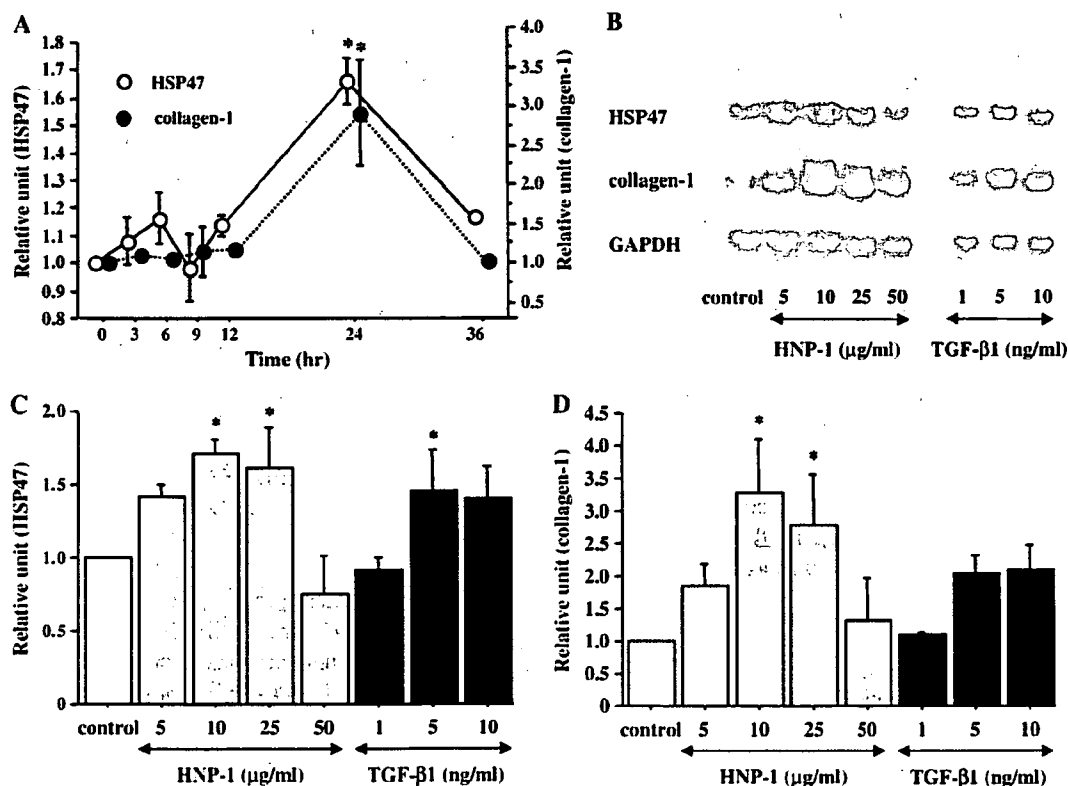


Fig. 2. Time course analysis of HSP47 and collagen-1 mRNA expression in NHLF treated with 10 µg/ml of HNP-1 by northern blotting (A). Representative autoradiographs of northern blot (B) showing mRNA expression of HSP47 and collagen-1 in NHLF after 24 h incubation with medium alone (control), 5, 10, 25 and 50 µg/ml of HNP-1 and 1, 5 and 10 ng/ml of TGF-β1. Results of densitometric analysis of bands on autoradiographs are shown in Fig. 2C (HSP47) and Fig. 2D (collagen-1). The density of the bands representing the mRNAs was compared to that of the GAPDH mRNA band in the same lane and shown as the resultant ratio. Values are mean±SEM of three experiments. * $p < 0.05$, compared with the control.

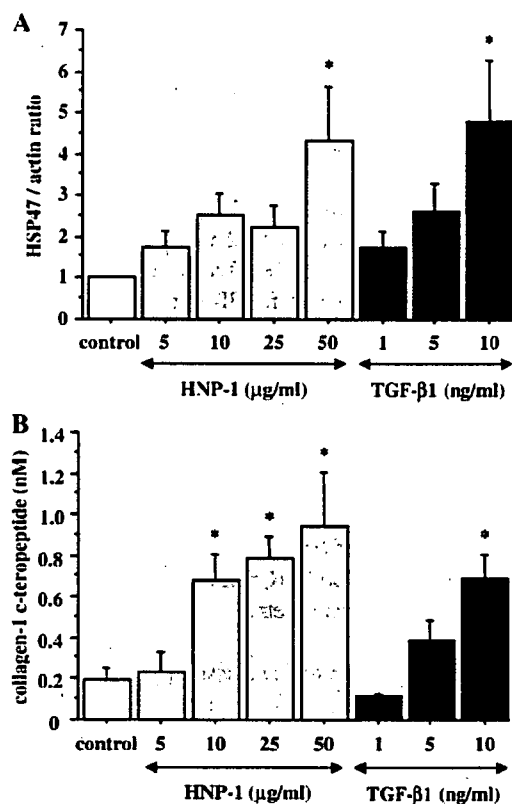


Fig. 3. HSP47 protein levels of NHLF analyzed by western blotting (A) and cell supernatant collagen-1 C-terminal telopeptide levels measured by ELISA (B) after 24 h incubation with medium alone (control), 5, 10, 25 and 50 µg/ml of HNP-1 and 1, 5 and 10 ng/ml of TGF-β1. The ratio (HSP47/actin) was detected by densitometry and normalized by control (control=1) in western blotting. Values are mean±SEM of four experiments. * $p < 0.05$, compared with the control.

CrossLaps for culture ELISA (Nordic Bioscience Diagnostics A/S, Herlev, Denmark). All measurements were performed in duplicate and the reported values are the mean of four measurements.

Statistical analysis

All values were expressed as mean±SEM. The minimum number of replicates for all measurements was at least three. Differences among various groups were compared by one-way ANOVA. Dunnett's test was used as the post hoc test for multiple comparisons. Significance was assumed at $p < 0.05$.

Results

Effect of HNP-1 on NHLF viability

We first examined the effect of HNP-1 (5, 10, 25, 50 and 100 µg/ml) on NHLF viability using Alamar Blue reduction assay. After 24-h treatment with HNP-1, the relative growth significantly increased at a concentration of 5 µg/ml compared with non-treated control cells, whereas HNP-1 concentrations more than 50 µg/ml significantly reduced the viability of NHLF (Fig. 1).

Effect of HNP-1 on HSP47 and collagen-1 mRNA expression

We examined the effects of HNP-1 (5, 10, 25, 50 µg/ml) and TGF-β1 (1, 5, 10 ng/ml) on the mRNA expression of HSP47 and collagen-1 in NHLF using northern blotting. We also examined the time course effect of HNP-1 (10 µg/ml) on this mRNA expression. The maximum response of HSP47 and collagen-1 to HNP-1 was observed after 24 h incubation (Fig. 2A). As shown in Fig. 2B,C, D, after 24-h treatment, HNP-1 at 10 and 25 µg/ml significantly increased the expression of HSP47 and collagen-1 mRNA compared with non-treated controls. Furthermore, 5 ng/ml of TGF-β1 significantly increased the expression of HSP47 mRNA (Fig. 2C), while TGF-β1 at all concentrations tested had no significant effect on the expression of collagen-1 mRNA (Fig. 2D).

HSP47 and collagen-1 protein production

We next examined the effects of HNP-1 and TGF-β1 on HSP47 and collagen-1 protein releases in NHLF using western blotting and ELISA, respectively. After 24-h treatment, the levels of HSP47 and collagen-1 proteins tended to increase dose-dependently by HNP-1 and TGF-β1. HNP-1 significantly increased the protein levels of HSP47 (at 50 µg/ml) and collagen-1 (at 10, 25 and 50 µg/ml). Furthermore, 10 ng/ml of TGF-β1 significantly increased the protein levels of HSP47 and collagen-1 (Fig. 3A and B).

MAPK signaling pathway and HNP-1-induced HSP47 protein production

To assess the involvement of MAPK signaling pathway in HNP-1 stimulation of NHLF, the cells were incubated with 10 µg/ml HNP-1 for 15 min to confirm the phosphorylation of ERK-1/2. The cells were incubated with inhibitor of MEK (PD98059) 1 h before the addition of HNP-1 for 24 h to assess the involvement of MAPK signaling pathway in HNP-1 enhanced HSP47 protein synthesis. Western blot analysis of ERK 1/2 phosphorylation demonstrated that 10 µg/ml HNP-1 stimulation increased the phosphorylated ERK 1/2 in NHLF (Fig. 4A, B), but western blot analysis of HSP47 demonstrated that 50 µM PD98059 did not block HSP47 protein production induced by 10 µg/ml HNP-1 (Fig. 4C, D).

Discussion

The major finding of this study was that HNP-1 (α-defensin) induced HSP47 and collagen-1 mRNA expression and production of their proteins in human lung fibroblasts. Low-dose (5 µg/ml) HNP-1 significantly induced relative cell growth but had minor effects on the expression of HSP47 and collagen-1, whereas HNP-1 at > 50 µg/ml was presumably cytotoxic to lung fibroblasts.

Neutrophils are thought to play an important role in the pathogenesis of variety inflammatory lung diseases through their derived products such as defensins and neutrophil elastase (Ashitani et al., 1998a,b, 2004; Ganz, 1987; Mukae et al., 2002; van Wetering et al., 1999; Yamanouchi et al., 1998). While

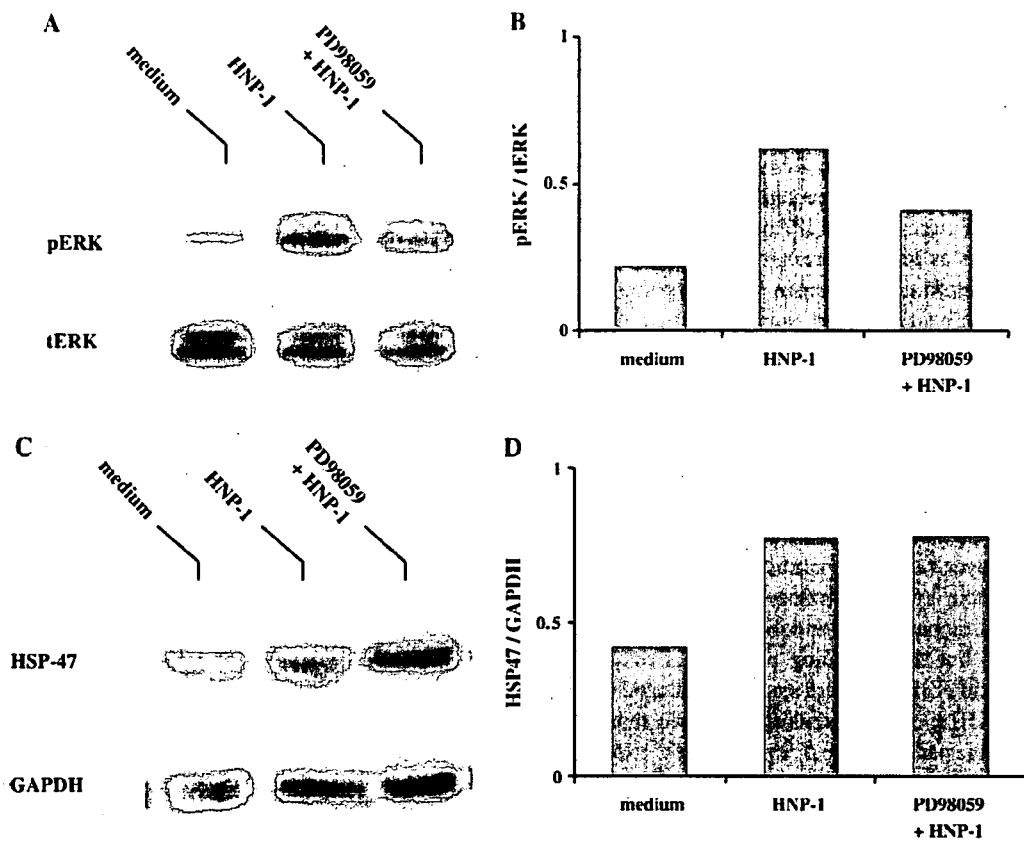


Fig. 4. (A) Activation of MAPK by HNP-1. NHLF were pre-incubated with 50 μ M PD98059 for 1 h before addition of 10 μ g/ml HNP-1 for 15 min. Phosphorylated ERK 1/2 (pERK) and total ERK 1/2 (tERK) were determined in cell lysates by Western blot (B). The ratio (pERK/tERK) was determined by densitometry. (C) Effect of inhibitor of MAPK signaling pathway on HSP47 protein synthesis induced by HNP-1. NHLF were pre-incubated with 50 μ M PD98059 for 1 h before addition of 10 μ g/ml HNP-1 for 24 h. HSP47 and GAPDH proteins were determined in cell lysates by western blot (D). The ratio (HSP47/GAPDH) was determined by densitometry. Data represent mean of two separate experiments, each performed in duplicate.

neutrophilic inflammation is regarded as an acute inflammatory response, the persistent presence of neutrophils seems to be associated with the fibrotic response (Sibille and Marchandise., 1993). Indeed, it was reported that BAL neutrophil counts correlated with treatment response (Turner-Warwick and Haslam, 1987), morphologic extent (Wells et al., 1998), rapidly progressive (Suga et al., 2000) and untreated active disease in IPF (Crystal et al., 1984). Furthermore, an *in vitro* study demonstrated that co-culture of fibroblasts and neutrophils significantly augmented fibroblast-mediated contraction of collagen gels (Skold et al., 1999), suggesting that the interaction between neutrophils and fibroblasts is closely associated with collagen deposition. However, the exact mechanism for this remains unclear.

The roles of α -defensins in inflammatory lung diseases are currently thought to be not only antimicrobial, but also modulation of inflammatory and immune response and wound repair (van Wetering et al., 1999). Murphy et al. showed that HNP-1 induces a dose-dependent increase in DNA synthesis in mouse lens epithelial cells and NIH 3T3 fibroblasts (Murphy et al., 1993). Oono et al. also demonstrated that synthetic HNP-1 enhanced the expression of pro- α 1(I) collagen in cultured human dermal fibroblasts (Oono et al., 2002). These results and our

present data indicate that α -defensins could promote directly the synthesis of fibrogenic molecules in fibroblasts. In our study, HNP-1 also induced HSP47 mRNA expression and its protein production in human lung fibroblasts. Overexpression of HSP47 has been described in experimental animal models of fibrosis, including murine bleomycin-induced pulmonary fibrosis (Ishii et al., 2003; Kakugawa et al., 2004), rat peritoneal sclerosis (Nishino et al., 2003) and carbon tetrachloride-induced rat liver cirrhosis (Masuda et al., 1994) and in patients with IPF (Kakugawa et al., 2005), suggesting the importance of HSP47 in collagen synthesis in various fibrotic disorders. Thus, the fibrotic effects of HNP-1 may be mediated not only through collagen production but also through HSP 47 expression, which may lead to excessive collagen synthesis. In this context, previous reports showed a strong positive staining for HNP-1 as well as HSP47 and type 1 procollagen in areas of active fibrosis (Kakugawa et al., 2005; Mukae et al., 2002), suggesting that α -defensins released by neutrophils in the alveolar septa could directly induce excessive deposition of extracellular matrix proteins regulating HSP47 and collagen-1 in patients with IPF.

HNP-1 concentrations used in this study were higher than those in BAL fluid of patients with IPF (Mukae et al., 2002). In

this context, we have previously measured the levels of HNP-1 in epithelial lining fluid (ELF) by using bronchoscopic microsampling method and BAL fluid in patients with pulmonary sarcoidosis. The levels of HNP-1 in ELF of 14 patients were about 100,000 times higher than those in BAL fluid (713 [154–1995] ng/ml versus 5 [0–37] pg/ml (median [range]), respectively) (unpublished data). Furthermore, in IPF patients, a strong positive staining for HNP-1 was found in areas of active fibrosis (Mukae et al., 2002). Therefore, α -defensin concentrations in the sequestered environment between stimulated neutrophil and target cells are suggested to reach more high and these doses might be significant effect in HSP47 and collagen-1 expressions of lung fibroblasts.

Aarbiou et al. (Aarbiou et al., 2002) showed that HNP mediated proliferation of A549 lung epithelial cells via the MAPK signaling pathway. In the present study, we showed that MAPK signaling pathway in NHLF was activated by HNP-1 stimulation, but specific MEK inhibitor (PD98059) did not block HSP47 protein synthesis induced by HNP-1 in NHLF. The MAPK signaling pathway is involved in the stimulation of HNP-1 in NHLF, but this signaling pathway is not the only pathway involved in HSP47 protein synthesis induced by HNP-1 stimulation in NHLF. Further studies of the phosphorylation of other signaling pathways and experiments using other specific inhibitors of each signaling pathway are required for better understandings of these events.

Acknowledgements

The authors thank A Yokoyama and S Tajiri for the technical support and Prof. M Nakazato (the Third Department of Internal Medicine, Faculty of Medicine, Miyazaki University) for the relevant advice. This study was supported in part by a research grant from the Ministry of Education, Science, Sports, and Culture of Japan.

References

- Aarbiou, J., Ertmann, M., van Wetering, S., van Noort, P., Rook, D., Rabe, K.F., Litvinov, S.V., van Krieken, J.H., de Boer, W.I., Hiemstra, P.S., 2002. Human neutrophil defensins induce lung epithelial cell proliferation in vitro. *Journal of Leukocyte Biology* 72, 167–174.
- Ashitani, J., Mukae, H., Nakazato, M., Ihi, T., Mashimoto, H., Kadota, J., Kohno, S., Matsukura, S., 1998a. Elevated concentrations of defensins in bronchoalveolar lavage fluid in diffuse panbronchiolitis. *European Respiratory Journal* 11, 104–111.
- Ashitani, J., Mukae, H., Nakazato, M., Taniguchi, H., Ogawa, K., Kohno, S., Matsukura, S., 1998b. Elevated pleural fluid levels of defensins in patients with empyema. *Chest* 113, 788–794.
- Ashitani, J., Mukae, H., Arimura, Y., Sano, A., Tokojima, M., Nakazato, M., 2004. High concentrations of alpha-defensins in plasma and bronchoalveolar lavage fluid of patients with acute respiratory distress syndrome. *Life Sciences* 75, 1123–1134.
- Boonars, K.A., Wagenaar, S.S., Mulder, P.G., van Velzen-Blad, H., van den Bosch, J.M., 1995. Relationship between cells obtained by bronchoalveolar lavage and survival in idiopathic pulmonary fibrosis. *Thorax* 50, 1087–1092.
- Crystal, R.G., Bitterman, P.B., Rennard, S.I., Hance, A.J., Keogh, B.A., 1984. Interstitial lung diseases of unknown cause. Disorders characterized by chronic inflammation of the lower respiratory tract (first of two parts). *New England Journal of Medicine* 310, 154–166.
- Ganz, T., 1987. Extracellular release of antimicrobial defensins by human polymorphonuclear leukocytes. *Infection and Immunity* 55, 568–571.
- Hunninghake, G.W., Kawanami, O., Ferrans, V.J., Young, R.C., Roberts Jr., W.C., Crystal, R.G., 1981. Characterization of the inflammatory and immune effector cells in the lung parenchyma of patients with interstitial lung disease. *American Review of Respiratory Disease* 123, 407–412.
- Ishii, H., Mukae, H., Kakugawa, T., Iwashita, T., Kaida, H., Fujii, T., Hayashi, T., Kadota, J., Kohno, S., 2003. Increased expression of collagen-binding heat shock protein 47 in murine bleomycin-induced pneumopathy. *American Journal of Physiology. Lung Cellular and Molecular Physiology* 285, L957–L963.
- Kakugawa, T., Mukae, H., Hayashi, T., Ishii, H., Abe, K., Fujii, T., Oku, H., Miyazaki, M., Kadota, J., Kohno, S., 2004. Pirfenidone attenuates expression of hsp47 in murine bleomycin-induced pulmonary fibrosis. *European Respiratory Journal* 24, 57–65.
- Kakugawa, T., Mukae, H., Hayashi, T., Ishii, H., Nakayama, S., Sakamoto, N., Yoshioka, S., Sugiyama, K., Mine, M., Mizuta, Y., Kohno, S., 2005. Expression of hsp47 in usual interstitial pneumonia and nonspecific interstitial pneumonia. *Respiratory Research* 6, 57.
- Masuda, H., Fukumoto, M., Hirayoshi, K., Nagata, K., 1994. Coexpression of the collagen-binding stress protein hsp47 gene and the alpha 1(I) and alpha 1 (III) collagen genes in carbon tetrachloride-induced rat liver fibrosis. *Journal of Clinical Investigation* 94, 2481–2488.
- Matsuoka, Y., Kubota, H., Adachi, E., Nagai, N., Marutani, T., Hosokawa, N., Nagata, K., 2004. Insufficient folding of type IV collagen and formation of abnormal basement membrane-like structure in embryoid bodies derived from hsp47-null embryonic stem cells. *Molecular Biology of the Cell* 15, 4467–4475.
- Mukae, H., Iiboshi, H., Nakazato, M., Hiratsuka, T., Tokojima, M., Abe, K., Ashitani, J., Kadota, J., Matsukura, S., Kohno, S., 2002. Raised plasma concentrations of alpha-defensins in patients with idiopathic pulmonary fibrosis. *Thorax* 57, 623–628.
- Murphy, C.J., Foster, B.A., Mannis, M.J., Selsted, M.E., Reid, T.W., 1993. Defensins are mitogenic for epithelial cells and fibroblasts. *Journal of Cellular Physiology* 155, 408–413.
- Nagai, N., Hosokawa, M., Itoharu, S., Adachi, E., Matsushita, T., Hosokawa, N., Nagata, K., 2000. Embryonic lethality of molecular chaperone hsp47 knockout mice is associated with defects in collagen biosynthesis. *Journal of Cell Biology* 150, 1499–1506.
- Nakai, A., Satoh, M., Hirayoshi, K., Nagata, K., 1992. Involvement of the stress protein hsp47 in procollagen processing in the endoplasmic reticulum. *Journal of Cell Biology* 117, 903–914.
- Nishino, T., Miyazaki, M., Abe, K., Furusu, A., Mishima, Y., Harada, T., Ozono, Y., Koji, T., Kohno, S., 2003. Antisense oligonucleotides against collagen-binding stress protein hsp47 suppress peritoneal fibrosis in rats. *Kidney International* 64, 887–896.
- Obayashi, Y., Yamadori, I., Fujita, J., Yoshinouchi, T., Ueda, N., Takahara, J., 1997. The role of neutrophils in the pathogenesis of idiopathic pulmonary fibrosis. *Chest* 112, 1338–1343.
- Oono, T., Shirafuji, Y., Huh, W.K., Akiyama, H., Iwatsuki, K., 2002. Effects of human neutrophil peptide-1 on the expression of interstitial collagenase and type I collagen in human dermal fibroblasts. *Archives of Dermatological Research* 294, 185–189.
- Pan, H., Halper, J., 2003. Regulation of heat shock protein 47 and type I procollagen expression in avian tendon cells. *Cell and Tissue Research* 311, 373–382.
- Schwartz, D.A., van Fossen, D.S., Davis, C.S., Helmers, R.A., Dayton, C.S., Burneister, L.F., Hunninghake, G.W., 1994. Determinants of progression in idiopathic pulmonary fibrosis. *American Journal of Respiratory and Critical Care Medicine* 149, 444–449.
- Sibille, Y., Marchandise, F.X., 1993. Pulmonary immune cells in health and disease: polymorphonuclear neutrophils. *European Respiratory Journal* 6, 1529–1543.
- Skold, C.M., Liu, X., Umino, T., Zhu, Y., Ohkuni, Y., Romberger, D.J., Spurzem, J.R., Heires, A.J., Rennard, S.I., 1999. Human neutrophil elastase augments fibroblast-mediated contraction of released collagen gels. *American Journal of Respiratory and Critical Care Medicine* 159, 1138–1146.
- Suga, M., Iyonaga, K., Okamoto, T., Gushima, Y., Miyakawa, H., Akaike, T., Ando, M., 2000. Characteristic elevation of matrix metalloproteinase

- activity in idiopathic interstitial pneumonias. *American Journal of Respiratory and Critical Care Medicine* 162, 1949–1956.
- Turner-Warwick, M., Haslam, P.L., 1987. The value of serial bronchoalveolar lavages in assessing the clinical progress of patients with cryptogenic fibrosing alveolitis. *American Review of Respiratory Disease* 135, 26–34.
- van Wetering, S., Sterk, P.J., Rabe, K.F., Hiemstra, P.S., 1999. Defensins: key players or bystanders in infection, injury, and repair in the lung? *Journal of Allergy and Clinical Immunology* 104, 1131–1138.
- Wells, A.U., Hansell, D.M., Haslam, P.L., Rubens, M.B., Cailles, J., Black, C.M., du Bois, R.M., 1998. Bronchoalveolar lavage cellularity: lone cryptogenic fibrosing alveolitis compared with the fibrosing alveolitis of systemic sclerosis. *American Journal of Respiratory and Critical Care Medicine* 157, 1474–1482.
- Yamanouchi, H., Fujita, J., Hojo, S., Yoshinouchi, T., Kamei, T., Yamadori, I., Ohtsuki, Y., Ueda, N., Takahara, J., 1998. Neutrophil elastase: alpha-1-proteinase inhibitor complex in serum and bronchoalveolar lavage fluid in patients with pulmonary fibrosis. *European Respiratory Journal* 11, 120–125.

C-Mannosylated peptides derived from the thrombospondin type 1 repeat enhance lipopolysaccharide-induced signaling in macrophage-like RAW264.7 cells

Eiji Muroi^{2,3,4,5}, Shino Manabe^{5,6}, Midori Ikezaki^{3,5},
Yoshishige Urata³, Shinichi Sato⁴, Takahito Kondo³,
Yukishige Ito^{5,6}, and Yoshito Ihara^{1,2,3,5,7}

³Department of Biochemistry and Molecular Biology in Disease, Atomic Bomb Disease Institute and ⁴Department of Dermatology, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki 852-8523, Japan; ⁵CREST, JST Kawaguchi 332-1102, Kawaguchi, Japan; ⁶RIKEN, Saitama 351-0198, Japan; and ⁷Department of Biochemistry, Wakayama Medical University, Wakayama 641-8509, Japan

Received on May 18, 2007; revised on June 20, 2007; accepted on June 24, 2007

C-Mannosylation is a unique type of glycosylation occurring at the first Trp (W) in the WXXW motif, which is found in the thrombospondin type 1 repeat (TSR) of proteins. However, the biological function of C-mannosylation is not fully understood. In this study, we investigated the effect of C-mannosylated TSR-derived peptides on lipopolysaccharide (LPS)-induced signaling in macrophage-like RAW264.7 cells. The cells were stimulated with LPS in the presence or absence of chemically synthesized peptides with or without C-mannose (e.g., (C-Man)-Trp-Ser-Pro-Trp [C-Man-WSPW], C-Man-W, WSPW, etc.), then the effects of the peptides on cellular viability and signaling were examined. We found a cytotoxic effect in the cells treated with LPS and C-Man-WSPW, but not in the cells solely treated with LPS or C-Man-WSPW. We also found that production of tumor necrosis factor- α (TNF- α) was upregulated more in response to LPS plus C-Man-WSPW, than in response to LPS plus WSPW or LPS alone. Among the LPS-induced signaling pathways that induce production of TNF- α , the activation of c-Jun N-terminal kinase (JNK) was greatly enhanced by LPS and C-Man-WSPW, and the production of TNF- α was suppressed by an inhibitor for JNK. Together, these results demonstrate a novel function of the C-mannosylated TSR-derived peptide motif, to promote LPS-induced JNK signaling, and this leads to an enhancement of cytotoxicity via the upregulation of TNF- α production.

Keywords: C-mannosylation/lipopolysaccharide/macrophage/thrombospondin

Introduction

C-Mannosylation is unique in that an α -mannose is directly bound to the indole C2 carbon atom of a Trp (W) residue through a C-C bond to produce C-mannosyl Trp (C-Man-W)

(Hofsteenge et al. 1994). C-Mannosylation occurs at the first Trp in the consensus amino acid sequence Trp-X-X-Trp (WXXW) in proteins (Furmanek and Hofsteenge 2000). The WXXW motif is found in the thrombospondin type 1 repeat (TSR), which is predicted to be a functional peptide module in various integral proteins such as thrombospondin-1 (TSP-1) (Tucker 2004). The motif is known to bind with heparin, heparan sulfate proteoglycans, and collagen, suggesting a functional significance in cell-cell interaction and/or cellular signaling. There are a number of examples of C-mannosylated proteins including ribonuclease 2 (Hofsteenge et al. 1994), Interleukin-12 (Doucey et al. 1999), complements (C6, C7, C8a, C8b, and C9) (Hofsteenge et al. 1999), properdin (Hartmann and Hofsteenge 2000), thrombospondin (Hofsteenge et al. 2001; De Peredo et al. 2002), F-spondin (Furmanek and Hofsteenge 2000), the erythropoietin receptor (Furmanek et al. 2003), mucins (MUC5AC and MUC5B) (Perez-Vilar et al. 2004). C-Mannosylation is thought to be carried out by a specific unidentified mannosyltransferase located in the microsomes. This suggests that C-mannosylation is involved in conventional glycosylation through the secretory pathway (Doucey et al. 1998).

Glycosylation has been revealed to have functional relevance to various cellular events including cell development, growth, differentiation, and death (Varki 1993; Dennis et al. 1999; Haltiwanger and Lowe 2004). However, the biological significance of C-mannosylation has yet to be fully investigated because a suitable methodology has not been established. In general, it is difficult to control the formation of glycoforms in cultured cells, which hampers progress in glycobiology and is a major obstacle to research into glycoprotein functions. One approach to conducting research in glycobiology is to generate well-defined synthetic glycopeptides or small glycoproteins using chemical or chemoenzymatic synthesis (Buskas et al. 2006). Since the discovery of C-mannosylated glycoproteins, several ways to obtain C²- α -D-C-Man-L-Trp building blocks have been reported (Manabe and Ito 1999; Nishikawa et al. 2001). Furthermore, Manabe et al. (2003) reported a route that differs from conventional approaches to C-glycosylation (Beau and Gallagher 1997). Recently, using a synthetic C-Man-W, we prepared a specific antibody against C-Man-W, then found that C-mannosylated proteins were expressed in mouse macrophage-like RAW264.7 cells, and the expression was upregulated under hyperglycemic conditions (Ihara et al. 2005).

To know whether C-mannosylation plays a functional role in macrophages, we focused on lipopolysaccharide (LPS)-induced signaling in macrophages, and investigated the effect of chemically synthesized C-mannosylated TSR-derived peptides (e.g., (C-Man)-Trp-Ser-Pro-Trp [C-Man-WSPW] [Figure 1]) on the signaling pathway in RAW264.7 cells. Here we report that C-mannosylated TSR-derived peptides modulate lipopolysaccharide (LPS)-induced signaling including the pathways involving

¹To whom correspondence should be addressed: Tel/Fax: +81-73-441-0628; e-mail: y-ihara@wakayama-med.ac.jp

²These authors contributed equally to this work.

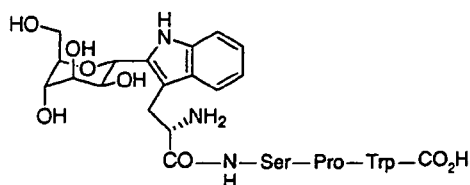


Fig. 1. The structure of C-mannosyl tryptophan in the TSR-derived peptide.

mitogen activating protein kinases (MAPKs) such as c-Jun N-terminal kinase (JNK), and enhance the cytotoxicity of LPS.

Results

C-Mannosylated TSR-derived peptides enhance LPS-induced cell damage in RAW264.7 cells

C-Mannosylated TSR-derived peptides and derivatives were chemically synthesized as described in Materials and methods. Cells were cultured for 48 h with different concentrations of LPS in the presence or absence of C-Man-WSPW or WSPW (10 μ M). The WSPW peptides, in which the first W has the potential to be C-mannosylated, are derived from TSR2 of human TSP-1, and the first W corresponds to Trp423 in the amino acid sequence (Lawler and Hynes 1986). Cell viability was examined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), as described in the methods (Figure 2A). Cell viability was little influenced by LPS between 0.1 and 5 μ g/mL. In the presence of C-Man-WSPW (10 μ M), viability was apparently reduced by LPS, especially at 1 μ g/mL. However, no enhancement of LPS-dependent cell damage was observed in the presence of WSPW (10 μ M). To further investigate the effect of C-Man-WSPW on LPS-induced cell damage, damaged cells were examined microscopically after staining with SYTOX Green, a noncell-permeable dye used to stain the nucleus (Figure 2B). Cells were cultured for 48 h with LPS (1 μ g/mL) in the presence or absence of C-Man-WSPW or WSPW (10 μ M). After the treatment with LPS, there was a slight enlargement of the cell body with or without C-Man-WSPW or WSPW. The cells stained with SYTOX Green were slightly observed in the cases with LPS plus WSPW or LPS alone. However, SYTOX Green-stained nuclei were observed more in the cells treated with LPS plus C-Man-WSPW, indicating that the plasma membrane was damaged in these cells. Together, these results suggest that C-Man-WSPW specifically induces damage in the cells treated with LPS.

Next, to see the dose-responsive effect of C-Man-WSPW on LPS-induced cell damage, cells were cultured for 48 h with LPS (1 μ g/mL) in the presence of various concentrations of C-Man-WSPW (0.5–10 μ M). Cell viability was examined as described above. The results showed that C-Man-WSPW enhanced the LPS-induced cell damage in a dose-dependent manner (Figure 2C). To further investigate the structural requirement of C-mannosylated peptides for enhancement of the damage due to LPS, cells were cultured for 48 h with or without LPS (1 μ g/mL) in the presence or absence of mannose, C-Man-W, C-mannosylated peptides with different structures (e.g., C-Man-WS, C-Man-WSP, C-Man-WSPW, C-Man-WSPWS, C-Man-WSG, and C-Man-WSK) or WSPW (10 μ M), then cell viability was examined. As shown in Figure 2D, C-mannosylated

peptides apparently enhanced the LPS-induced cell damage, especially in the cases of C-Man-WSP, C-Man-WSPW, and C-Man-WSPWS. C-Man-WSG and C-Man-WSK also moderately enhanced LPS-induced cell damage. On the other hand, C-Man-WS, C-Man-W, and mannose had a minor effect compared with other C-mannosylated peptides, and nonglycosylated WSPW had little effect as well. Furthermore, without LPS, cell viability was not reduced by mannose, C-Man-W, C-Man-WSPW, or WSPW (10 μ M). These results indicate that C-mannosylated peptides, which contain tripeptides such as WSP, effectively enhance LPS-induced damage in the cells.

Effect of C-mannosylated TSR-derived peptides on the binding of LPS to the cells

To investigate whether C-Man-WSPW influences the binding of LPS to RAW264.7 cells, the cells were incubated at 37°C for 20 min with FITC-conjugated LPS (LPS-FITC) (1 μ g/mL) in the presence or absence of WSPW or C-Man-WSPW (10 μ M) as described in the methods. After a wash with PBS, the binding of LPS-FITC to the cells was analyzed by flow cytometry (Figure 3A). The results showed that LPS-FITC was similarly bound to the cells in the presence of C-Man-WSPW or WSPW, indicating that C-Man-WSPW or WSPW did not directly influence the binding of LPS to the cells. Next, to determine the amount of WSPW or C-Man-WSPW bound to RAW264.7 cells, the cells were incubated with biotin, biotin-labeled WSPWC (WSPWC-biotin) or C-Man-WSPWC (C-Man-WSPWC-biotin) (10 μ M), and then the molecules bound to the cells were detected by flow cytometry after incubation with FITC-conjugated avidin (Figure 3B). The results showed that C-Man-WSPWC-biotin was bound to the cells, although the binding was weak. On the other hand, biotin and WSPWC-biotin showed less of a background signal. To further examine the interaction between C-mannosylated peptides and target proteins in the cell, the cells were incubated with biotin, WSPWC-biotin, or C-Man-WSPWC-biotin (10 μ M), and then the proteins bound to the biotin conjugates were detected, as described in the methods, by blot analysis using peroxidase-conjugated avidin after treatment with or without dithiobis[succinimidylpropionate] (DSP), a membrane-permeable crosslinker. As shown in Figure 3C, various proteins were bound to C-Man-WSPWC-biotin to a greater extent than those to WSPWC-biotin, under the conditions with DSP (arrows). The binding was not apparent under the conditions without DSP, although some non-specific binding of peroxidase-conjugated avidin to proteins was detected (arrow heads). These results suggest a specific but weak interaction between C-Man-WSPWC-biotin and the proteins.

Taken together, these results indicate that C-Man-WSPWC was predominantly bound to some target proteins of RAW264.7 cells, although it did not directly influence the binding of LPS to the cells.

C-Mannosylated TSR-derived peptides enhance LPS-induced production of TNF- α in the cells

LPS is cytotoxic due to the TNF- α or nitric oxide produced by the macrophages it stimulates (Guha and Mackman 2001). Thus, we focused on the effect of C-mannosylated TSR-derived peptides on the LPS-induced production of TNF- α and cell signaling. To investigate whether the C-mannosylated peptides influence the LPS-induced production of TNF- α , RAW264.7

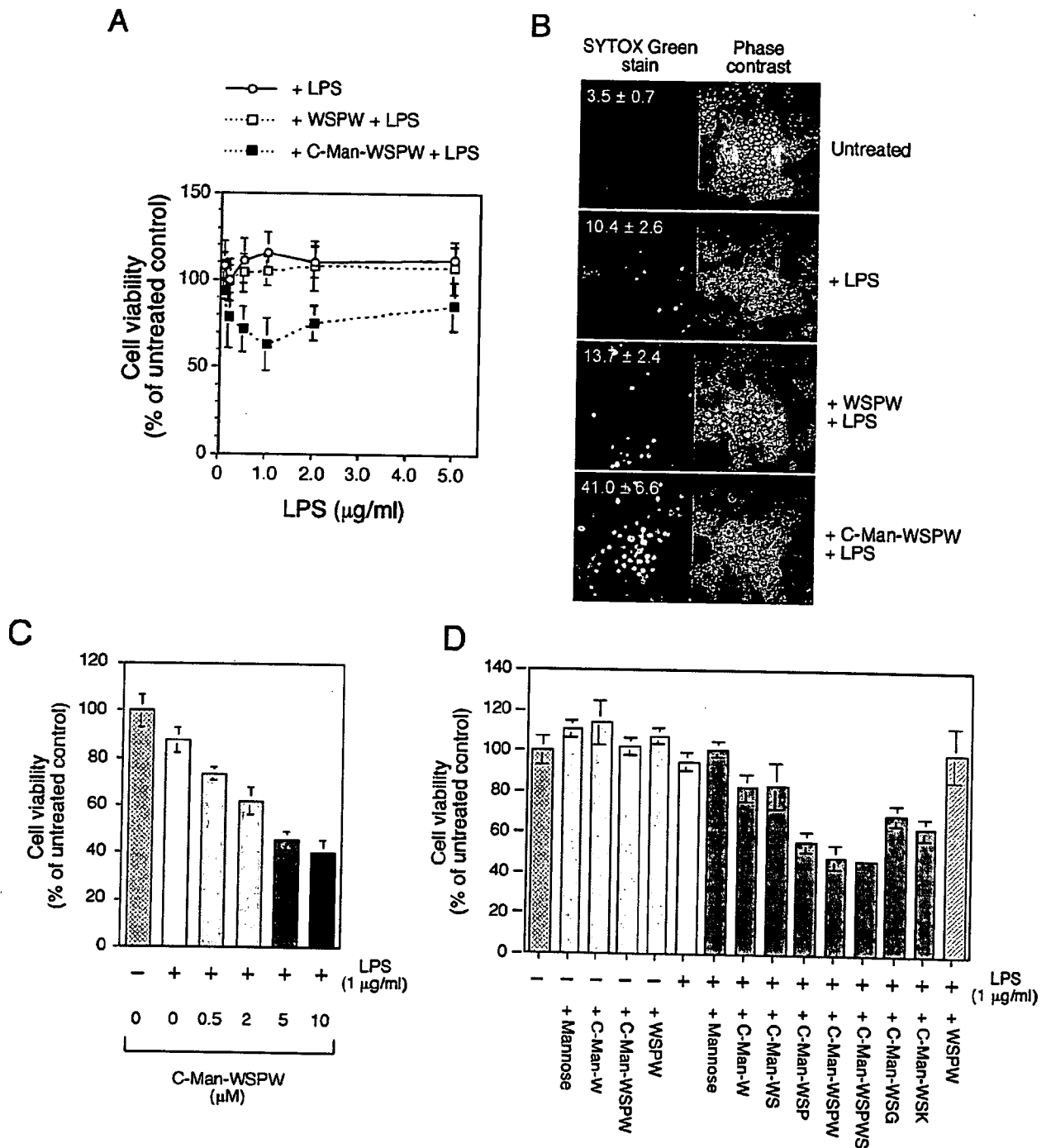


Fig. 2. C-Mannosylated TSR-derived peptides enhance the cytotoxicity of LPS in RAW264.7 cells. (A) RAW264.7 cells were cultured for 48 h in medium containing one of the several different concentrations of LPS in the presence or absence of C-Man-WSPW or WSPW (10 μM). Cell viability was examined by MTT assay, as described in Materials and methods. Each value represents the mean ± SD of four independent experiments. (B) Cells were treated as in A, then stained with SYTOX Green (1 μM) for 10 min. After fixation, signals were visualized by fluorescence microscopy with excitation at 485 nm and emission at 520 nm. Over 200 cells were counted to determine the proportion of SYTOX Green-positive cells (%). The values represent the means ± SD of three independent experiments. (C) Cells were cultured for 48 h with LPS (1 μg/mL) in the presence or absence of various concentrations of C-Man-WSPW (0.5–10 μM). Cell viability was examined by MTT assay, as described. (D) Cells were cultured for 48 h with or without LPS (1 μg/mL) in the presence or absence of mannose, C-Man-W, C-mannosylated peptides with different structures (C-Man-WS, C-Man-WSP, C-Man-WSPW, C-Man-WSPWS, C-Man-WSG, and C-Man-WSK), or WSPW (10 μM), and then cell viability was examined by MTT assay. Each value represents the mean ± SD of three to five independent experiments.

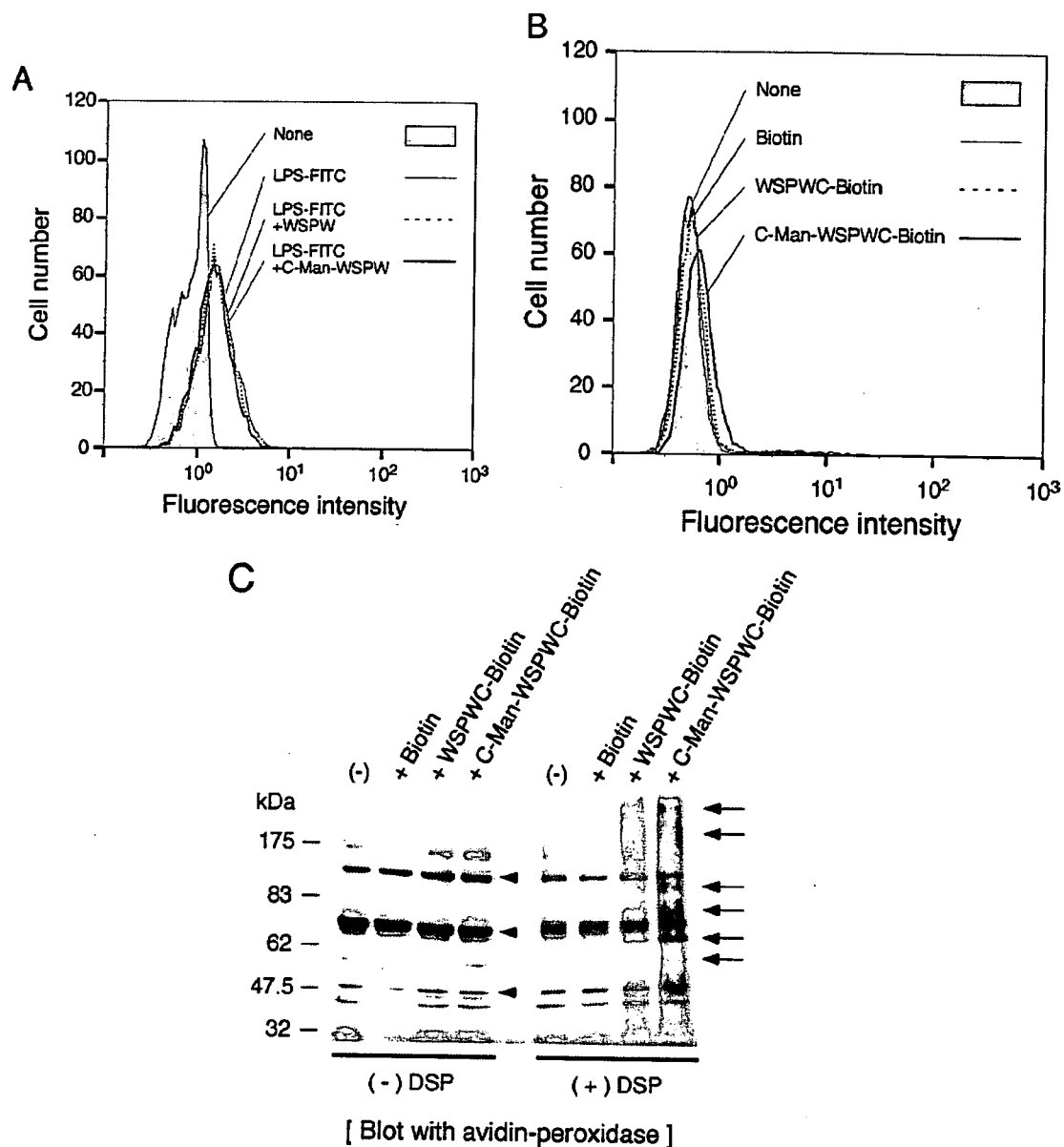


Fig. 3. Effect of C-Man-WSPW or WSPW on the binding of LPS to RAW264.7 cells. (A) Cells were incubated at 37°C for 20 min in medium containing LPS-FITC (1 µg/mL) in the presence or absence of C-Man-WSPW or WSPW (10 µM). After a wash with PBS, the binding of LPS-FITC to the cells was analyzed by flow cytometry, as described in Materials and methods. (B) Cells were incubated at 37°C for 20 min in PBS with biotin, WSPWC-biotin, or C-Man-WSPWC-biotin (10 µM). After a wash with PBS, the cells were blocked with 3% BSA in PBS, incubated with FITC-conjugated avidin for 15 min, and washed with PBS containing 1% BSA. After another wash, the binding of biotin conjugates to cells was analyzed by flow cytometry. (C) Cells were incubated with the biotin conjugates as described in B, and treated for 10 min with or without 1 mM DSP. After quenching with 1M Tris-HCl (pH 7.2), cells were washed with PBS, and subsequently harvested. The cells were lysed and protein samples were electrophoresed on 7.5% SDS-polyacrylamide gels under nonreducing conditions and then transferred to a nitrocellulose membrane. The membrane was blocked with 5% skim milk in TBS and then incubated at 4°C for 10 min with the peroxidase-conjugated avidin in TBS containing 0.1% Tween 20. After a wash with TBS containing 0.1% Tween 20, the blots were developed using an ECL chemiluminescence detection kit.

cells were treated with LPS (1 µg/mL) in the presence or absence of C-Man-WSPW or WSPW (10 µM), then the amount of TNF-α secreted from the cells was estimated by enzyme-linked immunosorbent assay (ELISA). As shown in Figure 4A, secretion of TNF-α increased in the culture medium following the treatments. In the case of LPS plus C-Man-WSPW, the secretion was especially enhanced at 1 and 2 h after the treatment, compared with the conditions with LPS plus WSPW or LPS alone.

The transcriptional expression of TNF-α was also examined by reverse transcription-polymerase chain reaction (RT-PCR) in the cells treated with LPS in the presence or absence of C-Man-WSPW or WSPW. The cells were treated for 1 and 2 h with LPS (1 µg/mL) in the presence or absence of C-Man-WSPW or WSPW (10 µM), then total RNA was extracted and subjected to RT-PCR. As shown in Figure 4B, the transcriptional level of TNF-α was enhanced more with LPS plus C-Man-WSPW than

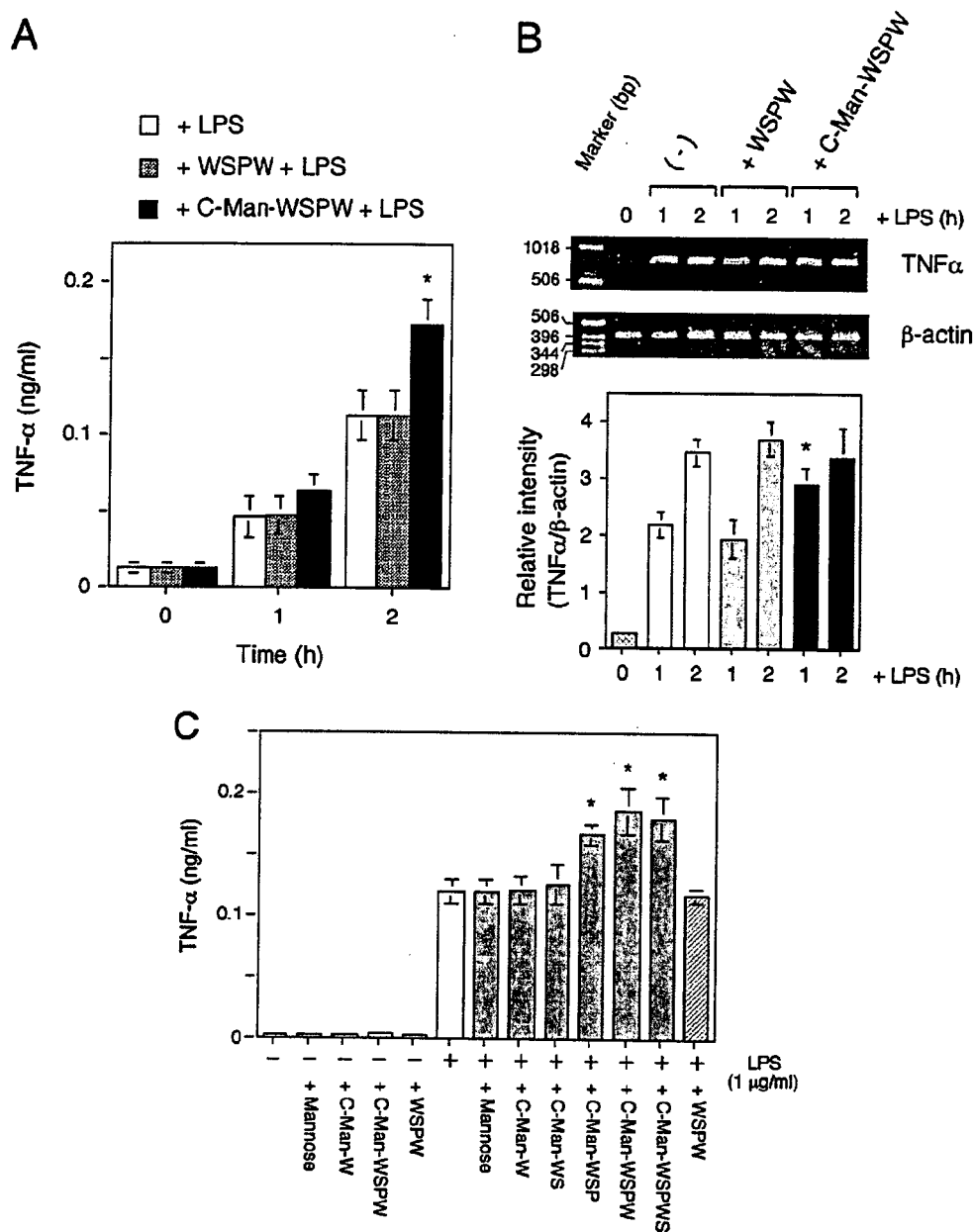


Fig. 4. C-Mannosylated TSR-derived peptides enhance the LPS-induced production of TNF- α in RAW264.7 cells. (A) Cells were plated in 96-well plates (10,000 cells/well), and cultured overnight. After being washed, the cells were stimulated for the periods indicated with LPS (1 μ g/mL) in the presence or absence of C-Man-WSPW or WSPW (10 μ M). After the incubation, the culture medium was collected, and the concentration of TNF- α in the sample was measured using a mouse TNF- α ELISA kit as described in Materials and methods. Each value represents the mean \pm SD of four independent experiments. * P < 0.05 vs. value for the cells treated with LPS or WSPW plus LPS. (B) Cells were stimulated for the periods indicated with LPS (1 μ g/mL) in the presence or absence of C-Man-WSPW or WSPW (10 μ M). Total RNA was prepared from the cells, and a RT-PCR analysis was performed to evaluate the transcriptional expression of TNF- α . The expression of β -actin was also shown as a control. The band intensity was estimated densitometrically, and the relative value of TNF- α to β -actin represents the mean \pm SE of three independent experiments (* P < 0.05 vs. value at same time point (1 h) for the cells treated with LPS or WSPW plus LPS). (C) Cells were cultured for 2 h with or without LPS (1 μ g/mL) in the presence or absence of mannose, C-Man-W, C-mannosylated peptides with different structures (C-Man-WS, C-Man-WSP, C-Man-WSPW, and C-Man-WSPWS), or WSPW (10 μ M), and then the TNF- α concentration in the medium was evaluated using a mouse TNF- α ELISA kit. Each value represents the mean \pm SD of three independent experiments (* P < 0.05 vs. value for the cells treated with LPS alone).

with LPS plus WSPW or LPS alone. Next, to investigate the structural requirement of C-mannosylated peptides for the enhancement of the LPS-induced production of TNF- α , cells were cultured for 2 h with LPS (1 μ g/mL) in the presence or absence of mannose, C-Man-W, C-mannosylated peptides with different

structures (e.g., C-Man-WS, C-Man-WSP, C-Man-WSPW, and C-Man-WSPWS), or WSPW (10 μ M), then the amount of TNF- α secreted was measured by ELISA as described. As shown in Figure 4C, C-mannosylated peptides, such as C-Man-WSP, C-Man-WSPW, and C-Man-WSPWS, apparently enhanced the

LPS-induced production of TNF- α , compared with LPS alone. C-Man-WSG and C-Man-WSK also similarly enhanced the LPS-induced production of TNF- α (data not shown). On the other hand, mannose, C-Man-W, and C-Man-WS had a minor effect compared with other C-mannosylated peptides, and nonglycosylated WSPW had very little effect. Furthermore, without LPS, the production of TNF- α was not triggered by mannose, C-Man-W, C-Man-WSPW or WSPW (10 μ M). These results indicate that C-mannosylated peptides, which contain tripeptides such as WSP, effectively enhance the LPS-induced production of TNF- α in RAW264.7 cells. Collectively, they indicate that LPS-induced expression of TNF- α is enhanced with C-mannosylated peptides such as C-Man-WSPW.

C-Mannosylated TSR-derived peptides enhance LPS-induced activation of JNK in the cells

LPS induces a variety of cellular signaling events, including the activation of NF- κ B and activation of the members of the MAPK family (Guha and Mackman 2001). The LPS signaling cascade leading to the production of TNF- α is controlled at the levels of both transcription of the TNF- α gene and translation of the mRNA. Transcriptional control of the gene is mediated primarily through the NF- κ B signaling pathway (Shakhov et al. 1990), and translation of the mRNA is regulated through the JNK pathway (Swantek et al. 1997). To investigate the effect of C-Man-WSPW on NF- κ B signaling with LPS, the transcriptional activity of NF- κ B was examined by conducting a luciferase reporter assay for NF- κ B as described in the methods. After transfection

with the luciferase vector for NF- κ B, the cells were treated with LPS (1 μ g/mL) in the presence or absence of WSPW or C-Man-WSPW (10 μ M), and reporter activity was measured (Figure 5A). The results showed that LPS-induced activation of NF- κ B was not influenced by C-Man-WSPW or WSPW. To further investigate the NF- κ B signaling with LPS, degradation of inhibitor of κ B α (I κ B α) was also examined by immunoblot analysis using specific antibodies, because degradation of I κ B α occurred during the activation of NF- κ B (Guha and Mackman 2001). The results showed that there was no difference in the LPS-induced degradation of I κ B α in the cells treated with or without C-Man-WSPW or WSPW (Figure 5B).

To investigate how C-Man-WSPW enhances the LPS-induced production of TNF- α , the activation status of several MAPKs was examined in the cells treated with LPS (1 μ g/mL) in the presence or absence of C-Man-WSPW or WSPW (10 μ M). The cells were treated with the reagents for given periods, then the phosphorylation status of MAPKs, such as extracellular signal-regulated kinase (ERK) 1/2, p38-MAPK, and JNK, was examined by immunoblot analysis using specific antibodies recognizing the phosphorylated form of each kinase. As shown in Figure 6A, B, and C, phosphorylation was induced in all tested MAPKs by the treatment with LPS, indicating that LPS upregulated MAPK signaling, and was consistent with previous findings related to LPS-induced MAPK signaling (Guha and Mackman 2001). The results also showed that the LPS-induced phosphorylation of MAPKs was little influenced by WSPW peptides. On the other hand, the LPS-induced phosphorylation was apparently enhanced by C-Man-WSPW especially

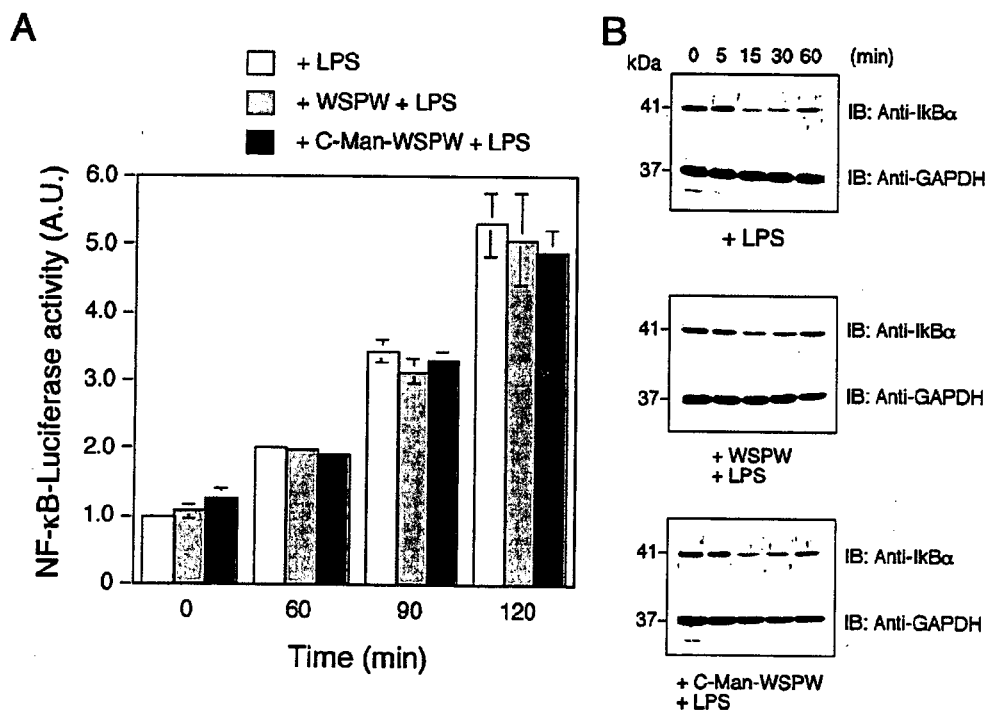


Fig. 5. Effect of C-Man-WSPW or WSPW on the NF- κ B signaling in RAW264.7 cells treated with LPS. (A) After 24 h of transfection with the luciferase vector for NF- κ B, the cells were incubated at 37°C for the periods indicated in medium containing LPS (1 μ g/mL) in the presence or absence of C-Man-WSPW or WSPW (10 μ M), and the reporter activity was measured as described in the methods. (B) Cells were incubated at 37°C for the periods indicated in medium containing LPS (1 μ g/mL) in the presence or absence of C-Man-WSPW or WSPW (10 μ M). Degradation of I κ B α was examined with samples of cell lysate by immunoblot analysis using specific antibodies as described in Materials and methods.

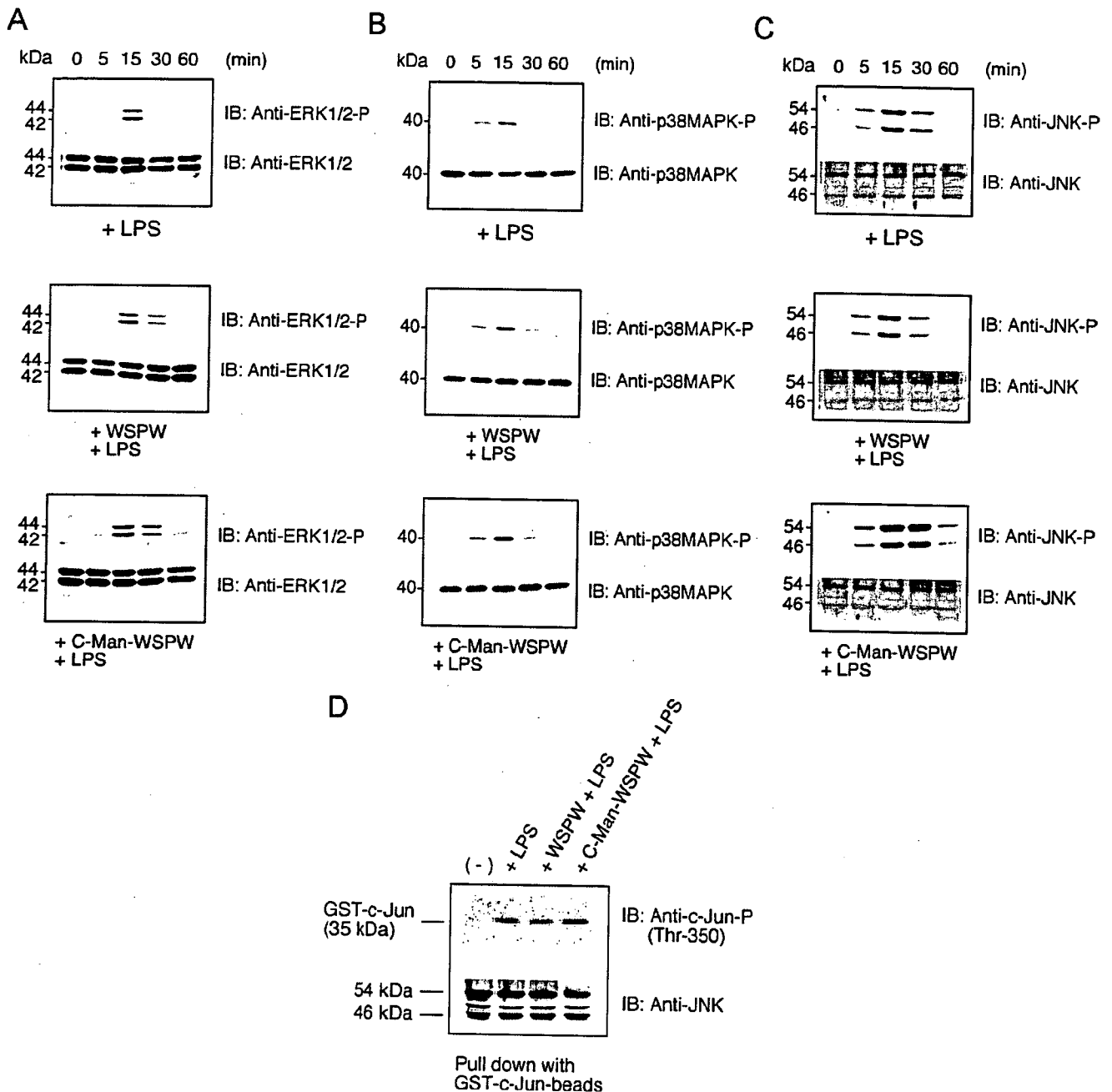


Fig. 6. Effect of C-Man-WSPW or WSPW on the MAPK signaling in RAW264.7 cells treated with LPS. A, B, C, and cells were treated with LPS, LPS plus WSPW, or LPS plus C-Man-WSPW, as described in Figure 5B. The phosphorylation status of MAPKs, such as ERK1/2 (Thr-202/Tyr-204) (A), p38-MAPK (Thr-180/Tyr-182) (B), and JNK (Thr-183/Tyr-185) (C), was examined with samples of cell lysate by immunoblot analysis using specific antibodies as described in Materials and methods. (D) The activity of JNK was examined, as described in the methods using GST-c-Jun as a substrate in the samples prepared from cells treated at 37°C for 15 min with LPS (1 μ g/mL) in the presence or absence of C-Man-WSPW or WSPW (10 μ M).

in the case of JNK, although slight enhancement was observed in the cases of ERK1/2 and p38-MAPK. The phosphorylation of MAPKs was not induced in the cells solely treated with WSPW or C-Man-WSPW (data not shown), indicating that LPS was required to upregulate all of the MAPK signaling. To confirm the enhanced activation of JNK in the cells treated with LPS plus C-Man-WSPW, the activity of JNK was examined using GST-c-Jun fusion protein as a substrate in samples prepared from cells

treated for 15 min with LPS (1 μ g/mL) in the presence or absence of C-Man-WSPW or WSPW (10 μ M). As shown in Figure 6D, phosphorylation of GST-c-Jun was upregulated more in the cells treated with LPS plus C-Man-WSPW, than those treated with LPS plus WSPW or LPS alone. This indicates that JNK activity is specifically enhanced by LPS plus C-Man-WSPW. Collectively, these results indicate that LPS-induced JNK signaling was upregulated by C-Man-WSPW, but not by WSPW,

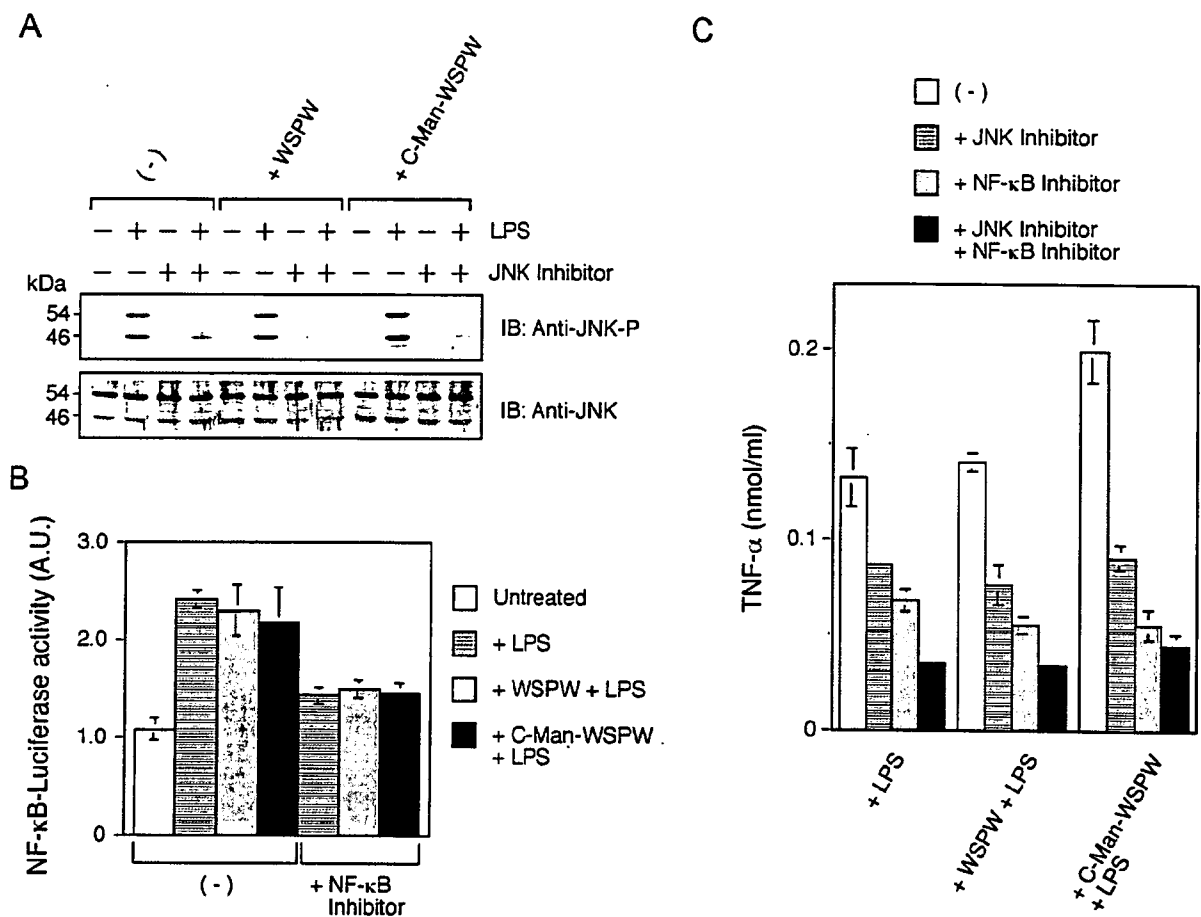


Fig. 7. JNK and NF-κB contribute to the LPS-induced production of TNF-α in RAW264.7 cells in the presence or absence of C-Man-WSPW or WSPW. (A) Cells were pretreated at 37°C for 10 min with the JNK inhibitor (SP600125, 10 μM), then treated for 15 min with LPS (1 μg/mL) in the presence or absence of C-Man-WSPW or WSPW (10 μM). The activation status of JNK was examined in the cell lysates by immunoblot analysis using the antiphospho-JNK (Thr183/Tyr-185) antibody as described in Materials and methods. (B) After 24 h of transfection with the luciferase vector for NF-κB, the cells were pretreated at 37°C for 10 min with a NF-κB activation inhibitor (10 μM), then treated for 90 min with LPS (1 μg/mL) in the presence or absence of C-Man-WSPW or WSPW (10 μM). The activation status of NF-κB was examined in the cell lysates by luciferase reporter assay as described in the methods. (C) Cells were pretreated at 37°C for 10 min with or without JNK inhibitor and/or the NF-κB activation inhibitor, and treated for 2 h with LPS (1 μg/mL) in the presence or absence of C-Man-WSPW or WSPW (10 μM), as described above. Then, amount of TNF-α released into the medium was estimated by ELISA for TNF-α.

despite that LPS-induced NFκB signaling was little influenced by C-Man-WSPW or WSPW.

JNK signaling pathway is involved in enhancement of LPS-induced upregulation of TNF-α by C-Man-WSPW

To investigate whether the signaling pathways of JNK and/or NF-κB are involved in the LPS-induced production of TNF-α, levels of TNF-α were examined in cells treated with or without LPS, LPS plus WSPW, and LPS plus C-Man-WSPW in the presence or absence of specific signaling inhibitors. SP600125 (a JNK Inhibitor) and a NF-κB activation inhibitor were used to inhibit the activities of JNK and NF-κB, respectively. In Figure 7A, the cells were pretreated for 10 min with the JNK inhibitor (10 μM), then treated for 15 min with LPS (1 μg/mL) in the presence or absence of C-Man-WSPW or WSPW (10 μM). The activation status of JNK was examined in the cell lysates by immunoblot analysis using the anti-phospho-JNK antibody as described. The results showed

that the phosphorylation of JNK was significantly suppressed by the JNK inhibitor in all cases tested. In Figure 7B, after 24 h of transfection with the luciferase vector for NF-κB, the cells were pretreated for 10 min with the NF-κB activation inhibitor (10 μM), then treated for 90 min with LPS (1 μg/mL) in the presence or absence of C-Man-WSPW or WSPW (10 μM). The activation status of NF-κB was examined in the cell lysates by luciferase reporter assay as described. The results showed that the level of transcriptional activity for NF-κB was similarly enhanced by LPS with or without WSPW or C-Man-WSPW, but was suppressed by the inhibitor to approximately 60% of each level of NF-κB activity without the inhibitors. The cells were pretreated for 10 min with or without the JNK inhibitor and/or NF-κB activation inhibitor, and treated for 2 h with LPS, LPS plus WSPW, or LPS plus C-Man-WSPW (Figure 7C). Then, the amount of TNF-α released into the medium was estimated by an ELISA for TNF-α. The results showed that LPS-induced production of TNF-α was suppressed by the inhibitors for JNK and/or NF-κB with or without WSPW or

C-Man-WSPW. Although the suppression was enhanced more with the JNK inhibitor plus NF- κ B activation inhibitor, the production of TNF- α was not completely suppressed to the level of the untreated background (see Figure 4A).

Taken together, these results demonstrate that both the JNK and NF- κ B pathways are important to the production of TNF- α induced in all cases with LPS, LPS plus WSPW, and LPS plus C-Man-WSPW, suggesting that enhanced JNK signaling in the cells treated with LPS plus C-Man-WSPW may be part of the signaling pathway responsible for upregulating the production of TNF- α .

C-Mannosylated TSR-derived peptides enhance LPS-induced phosphorylation of TAK1 in RAW264.7 cells

In terms of signaling pathways for LPS-induced TNF- α production, we found that the activation of JNK was specifically upregulated by LPS in the presence of C-Man-WSPW. To further investigate how C-Man-WSPW enhances LPS-induced activation of JNK, we focused on the upstream signaling molecules, such as interleukin-1 receptor-associated kinase 1 (IRAK1) and transforming growth factor- β (TGF- β)-activated kinase 1 (TAK1). IRAK1 is one of the upstream serine/threonine kinases activated by LPS through Toll-like receptor 4 (TLR4) (Janssens and Beyaert 2003). The activation of IRAK1 occurs with a concomitant self-phosphorylation of serine or threonine (Kollewe et al. 2004). In Figure 8A, cells were treated with LPS (1 μ g/mL) in the presence or absence of C-Man-WSPW or WSPW

(10 μ M). The activation status of IRAK1 was examined in the cell lysates by immunoblot analysis using the anti-phospho-IRAK1 (Thr-209) antibody, as described. The results showed that the pattern of phosphorylation of IRAK1 did not differ with LPS, LPS plus WSPW, or LPS plus C-Man-WSPW, suggesting that the activation of IRAK1 was little influenced by WSPW or C-Man-WSPW. On the other hand, TAK1, a MAPK kinase kinase (MAPKKK) activated by LPS through TLR4 signaling, is also involved in the LPS-induced signaling pathways to activate NF- κ B and MAPKs, such as JNK (Irie et al. 2000; Chen et al. 2002; Silverman et al. 2003) (Figure 8B). The activation of TAK1 occurs with a concomitant autophosphorylation of serine or threonine (Kishimoto et al. 2000; Singhirunnusorn et al. 2005). The activation status of TAK1 was also examined in the cell lysates by immunoblot analysis using the anti-phospho-TAK1 (Thr-187) antibody, as described in Materials and methods. The results showed that the phosphorylation of TAK1 was significantly enhanced by LPS plus C-Man-WSPW, but not by LPS plus WSPW or LPS alone (Figure 8B).

Taken together, C-Man-WSPW or WSPW did not influence the LPS-induced activation of IRAK1, a pivotal LPS-induced kinase located upstream of TAK1, which was consistent with the finding that the quantity of LPS-FITC bound to the cells was not affected by C-Man-WSPW or WSPW. However, C-Man-WSPW specifically upregulated LPS-induced activation of TAK1, a pivotal MAPKKK for LPS-induced activation of MAPK pathways. This was consistent with enhanced LPS-induced activation of MAPKs such as JNK by C-Man-WSPW,

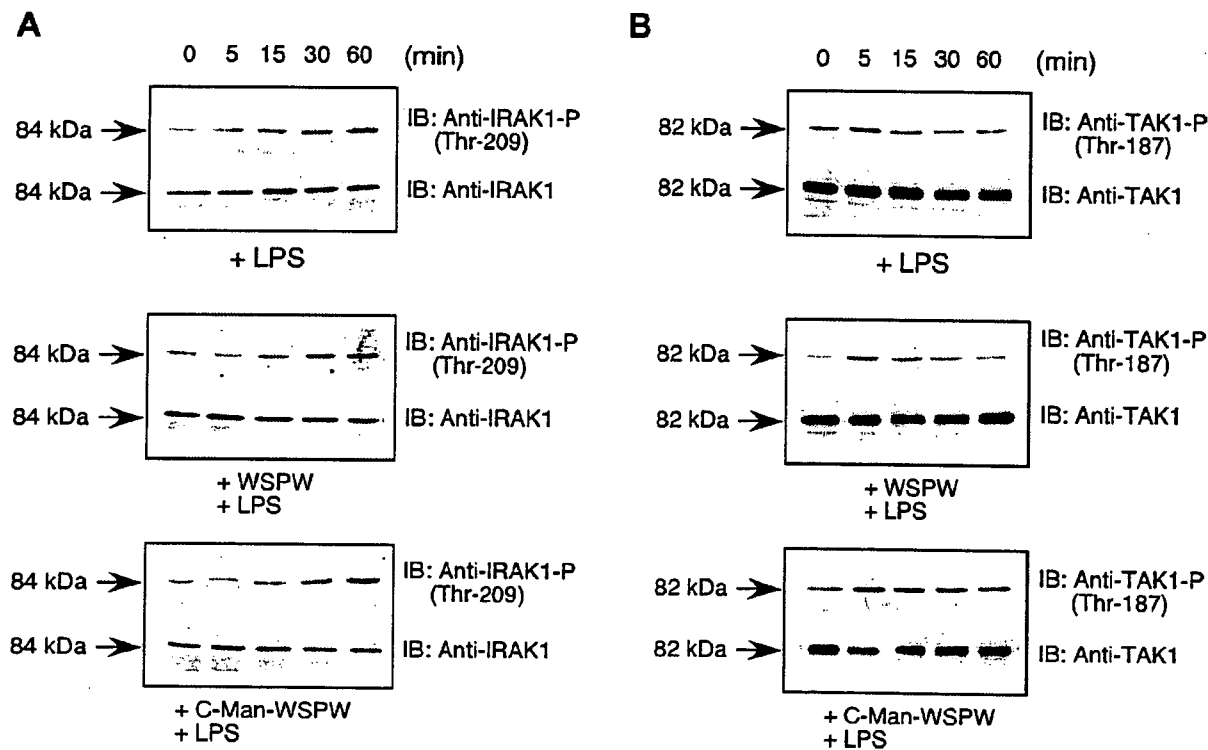


Fig. 8. LPS-induced phosphorylation of TAK1 is highly upregulated in RAW264.7 cells treated with C-Man-WSPW. (A) Cells were treated with LPS, LPS plus WSPW, or LPS plus C-Man-WSPW, as described in Figure 5B. The activation status of IRAK1 was examined in the cell lysates by immunoblot analysis using the antiphospho-IRAK1 (Thr-209) antibody as described in the methods. (B) Cells were treated with LPS, LPS plus WSPW, or LPS plus C-Man-WSPW, as described above. The activation status of TAK1 was also examined in the cell lysates by immunoblot analysis using the antiphospho-TAK1 (Thr-187) antibody, as described in Materials and methods.

resulting in increased production of TNF- α and further LPS-induced cell damage.

Discussion

In the present study, to know how C-mannosylation is involved in the functions of macrophages, we chemically synthesized C-mannosylated peptides such as C-Man-WSPW derived from the TSR motif of human TSP-1, and investigated their effect on LPS-induced signaling in macrophage-like RAW264.7 cells.

LPS, a component of the outer cell membrane of gram-negative bacteria, is a strong stimulator of macrophage/monocytes and shows a clearly inflammatory effect. It is also known that LPS is toxic to several types of cells, such as macrophages and endothelial cells, and that LPS is involved in the pathology of sepsis and septic shock (Akira 2001; Bannerman and Goldblum 2003). In this study, we found that C-mannosylated peptides enhanced LPS-induced damage in RAW264.7 cells, especially in the cases of C-Man-WSP, C-Man-WSPW, and C-Man-WSPWS. However, C-Man-WS, C-Man-W, and mannose had a minor effect compared with other C-mannosylated peptides, and nonglycosylated WSPW also had little effect. These results strongly suggest that C-mannosylated peptides, which contain tripeptides such as WSP, effectively enhance LPS-induced damage, and also that C-mannosylation confers a specific function on WSPW peptides which is commonly seen in the TSR motif of cellular proteins. However, it is not clear yet whether these C-mannosylated peptides can be naturally generated in the cell. In addition, to test functional importance of Pro in C-Man-WSP, we prepared C-Man-WSG and C-Man-WSK, in which Pro was replaced by Gly and Lys, respectively, and examined the effect of the C-mannosylated tripeptides on LPS-induced cell damage. As shown in Figure 2D, both C-Man-WSG and C-Man-WSK moderately enhanced LPS-induced cell damage, suggesting that the effect seems not necessarily dependent on Pro in the specific sequence of WSP.

A variety of C-mannosylated proteins have been identified, and some of these proteins, such as TSP, F-spondin, Interleukin-12, complements, and mucins, mainly function in the extracellular spaces (Furmanek and Hofsteenge 2000). Recently, it has been reported that TSP-1 and 2 are cleaved by ADAMTS1 (A Disintegrin And Metalloprotease with Thrombospondin 1), resulting in the production of functional antiangiogenic peptides (Lee et al. 2006). Furthermore, C-Man-W was detected in human serum or urine from patients of renal diseases (Takahira et al. 2001), suggesting that C-Man-W could be a novel biomarker for renal dysfunction. These results suggest that some of the functional C-mannosylated peptides might be generated in vivo by proteolytic degradation of C-mannosylated proteins. However, it is not clear yet what endogenous C-mannosylated peptides or proteins enhance LPS-induced signaling in macrophages. Thus, further investigation is required to identify such endogenous proteins bearing the C-mannosylated peptides in vivo.

In this study, we showed that the enhanced LPS-induced signaling with C-Man-WSPW was not simply due to the enhanced binding of LPS to cells treated with LPS and C-Man-WSPW, suggesting that the cellular binding of LPS was not directly affected by C-Man-WSPW. On the other hand, we found that the binding of C-Man-WSPWC-biotin to cells was greater than that of biotin or WSPWC-biotin on flow cytometry. In addition, under the conditions with DSP, we found that C-Man-WSPWC-

biotin was predominantly bound to some specific proteins of RAW264.7 cells, compared with the lower binding of biotin or WSPWC-biotin. Together, these results strongly suggest that C-Man-WSPW peptides exert an enhancing effect on LPS-induced cytotoxicity by their binding to some specific target proteins in cells, but not by affecting LPS binding to the cells.

The inflammatory effects of LPS are mediated through several secreted factors, such as TNF- α (Guha and Mackman 2001) and nitric oxide (Nathan 1992). We also found that C-Man-WSPW apparently enhanced the production of TNF- α in cells treated with LPS, compared to those treated with LPS plus WSPW or LPS alone. LPS starts the signal transduction by engaging itself with cell surface TLR4 via sequential binding with LPS-binding protein and CD14 (Guha and Mackman 2001). LPS has been shown to initiate multiple intracellular signaling events, such as the activation of NF- κ B, and three distinct MAPKs (i.e., ERK1/2, p38-MAPK, and JNK), and these pathways are involved in the LPS-induced upregulation of TNF- α production. In this study, we found that JNK was significantly activated in the cells treated with LPS plus C-Man-WSPW, compared with the cells treated with LPS plus WSPW or LPS alone, although other kinases such as ERK1/2 and p38-MAPK were also slightly upregulated in the activation by C-Man-WSPW plus LPS. On the other hand, the LPS-induced activation of NF- κ B was little influenced by C-Man-WSPW or WSPW. Wilson et al. (1999) reported that TSP-1 or TSP-1-derived peptides containing a WSXW motif enhanced CD3-induced activation of MAPK signaling including JNK signaling in T lymphocytes. In addition, Jimenez et al. (2001) reported that TSP-1 inhibits angiogenesis in corneal neovascularization via the activation of JNK signaling. However, it is not clear how the WXXW motif in the TSR is involved in the signaling to JNK.

The proximal signaling molecules involved in the LPS-induced activation of MAPKs have not been fully identified. LPS is thought to activate ERK1/2 through the Ras/Raf-1/MAPKK pathway, p38-MAPK through the activation of MAPK/ERK kinase (MEK)-3, and JNK through the MEKK-1/MEK4 pathway (Swanek et al. 1997; van der Bruggen et al. 1999). In this study, we focused on the upstream signaling of JNK, and examined the effect of C-Man-WSPW on the activation status of some upstream kinases induced by LPS. IRAK1 is one of the upstream kinases associated with the LPS-specific receptor, TLR-4, and is phosphorylated by LPS to be activated (Li et al. 2000). Unexpectedly, LPS-induced phosphorylation of IRAK1 was not influenced by C-Man-WSPW or WSPW, which was consistent with the finding that both C-Man-WSPW and WSPW did not influence the binding of LPS to cells. However, these results suggested that C-Man-WSPW indirectly acts on some intermediate in the LPS-induced signaling pathways to JNK. Next, we examined TAK1, a MAPKKK mainly involved in LPS-induced JNK signaling (Chen et al. 2002; Silverman et al. 2003), and found that LPS-induced phosphorylation of TAK1 was apparently enhanced by C-Man-WSPW but not by WSPW. This suggests that C-Man-WSPW influenced LPS-induced signaling to activate JNK at the level of TAK1 activity, although how the phosphorylation of TAK1 was upregulated by C-Man-WSPW is not known. Although TAK1 is located downstream of the pathway of LPS/TLR4/IRAK1/TNF- α receptor-associated factor (TRAF6) that activates JNK, an alternative pathway for the activation of TAK1 has been identified in cells expressing latent membrane protein 1 (LMP1) derived from Epstein-Barr

virus (Wan et al. 2004). LMP1 does not require myeloid differentiation factor 88 (MYD88), IRAK1, and IRAK4 to engage TRAF6, and selectively utilize TRAF6, TAK1/TAK1-binding protein, and JNK kinases 1 and 2 to activate JNK. Very recently, Uemura et al. (2006) demonstrated that TAK1 is involved in the LMP1 complex and is essential for activation of JNK but not of NF- κ B. This suggests that the LPS signaling pathway can be influenced by other modulators, which are not directly involved in the authentic LPS/TLR-4/TRAF6/IRAK/TAK1 pathways, implying that C-mannosylated TSR-derived peptides may act as another modulator of LPS-induced MAPK signaling by mimicking the functions of modulators such as LMP1, although the precise mechanism for the signal transduction by C-Man-WSPW is yet to be clarified.

In macrophages/monocytes, there is a variety of mannose recognizing proteins included in the mannose receptor family (East and Isacke 2002; McGreal et al. 2005). The mannose receptor family is a subgroup of the C-type lectin superfamily and comprises four members; the mannose receptor (MR), the M-type phospholipase A2 receptor, DEC-205 (CD205), and Endo-180. Among the members, MR and Endo-180 have the capacity to bind carbohydrates such as mannose, fucose, *N*-acetylglucosamine, etc., in a Ca²⁺-dependent manner via their carbohydrate recognition domains. On the other hand, among the C-type lectin receptors, Dectin-1, which recognizes β -linked glucans from fungi, is known to work in collaboration with TLR2 to facilitate inflammatory responses (Brown et al. 2003; Gantner et al. 2003). This also suggests a possible regulation of TLR-related signaling by C-mannosylated peptides via some co-lateral regulatory pathways. Hence, another question raised is whether the C-mannosylated peptides also modulate other receptor signaling pathways in macrophages (i.e., TLR2, TLR6, TLR9, interleukin-1 receptor, and TNF- α receptor), compared with the LPS/TLR4 pathway. Nishikawa et al. (2004) reported that C-Man-W is not recognized *in vitro* by conventional mannose-binding lectins such as concanavaline A and mannose-binding lectin-C, a lectin abundant in mouse serum. However, it is not clear yet what specific molecule binds to C-Man-W and C-mannosylated peptides. Further investigation to clarify the specific receptors recognizing the motif-containing C-mannosylated peptides is required.

In conclusion, C-mannosylated TSR-derived peptides have an enhancing effect on the cytotoxicity of LPS by increasing the production of TNF- α through enhanced activation of JNK. This study demonstrates a novel regulatory function of the C-mannosylated TSR-derived peptide motif in the innate immunity of macrophages, and also suggests unrevealed functions of C-mannosylated proteins or peptides in macrophages.

Materials and methods

Materials

Antibodies against ERK1/2, phospho-ERK1/2 (Thr-202/Tyr-204), p38-MAPK, phospho-p38MAPK (Thr-180/Tyr-182), JNK, phospho-JNK (Thr-183/Tyr-185), TAK1, phospho-TAK1 (Thr-187), IRAK1, phospho-IRAK1 (Thr-209), and I κ B α were purchased from Cell Signaling Technology (Beverly, MA). Peroxidase-conjugated secondary antibodies against IgG of rabbit and mouse were from Dako (Glostrup, Denmark). LPS from *Escherichia coli* O55:B5 and fluorescein isothio-

cyanate (FITC)-conjugated LPS (LPS-FITC) were obtained from Sigma-Aldrich (St. Louis, MO). Nuclear factor- κ B (NF- κ B) activation inhibitor [i.e., 6-amino-4-(4-phenoxyphenylethylamino)quinazoline] was from Calbiochem. DSP was from Pierce Biotechnology (Rockland, IL). The other reagents used in the study were all of high grade, from Sigma-Aldrich or Wako pure chemicals (Osaka, Japan).

Chemical synthesis of C-mannosylated peptides and derivatives

C²- α -D-C-Mannosylpyranosyl-L-tryptophan (C-Man-W) and C-mannosylated TSR-derived peptides (C-Man-WS, C-Man-WSP, C-Man-WSG, C-Man-WSK, C-Man-WSPW, and C-Man-WSPWS) were synthesized essentially, as described previously (Manabe and Ito 1999; Manabe et al. 2003). WSPW peptides, in which the first W has the potential to be C-mannosylated, are derived from TSR2 of human TSP-1, and the first W corresponds to Trp423 in the amino acid sequence (Lawler and Hynes 1986; NCBI Accession number NP_003237) (Figure 1). Silica gel 60N (spherical, neutral, Kanto Chemical Co., Inc, Tokyo) was used for flash column (40–100 μ M) and open column (100–200 μ M) chromatography. SephadexTM LH-20 (GE Healthcare Biosciences, Buckinghamshire, UK) was used for size-exclusion chromatography. Silica gel 60 F₂₅₄ (E. Merck, Whitehouse Station, NJ) was used for analytical thin-layer chromatography. MALDI-TOF MS spectra were measured with a Shimadzu AXIMA-CFR using 2,5-dihydrobenzoic acid or α -cyano-4-hydroxycinnamic acid as a matrix. ¹H-NMR (nuclear magnetic resonance) spectra were recorded at ambient temperature (23–24°C) in CDCl₃, CD₃OD or DMOS using JEOL EX 400 MHz spectrometer. A Waters C18 SepPack Cartridge was used for reverse phase column chromatography (Waters, Milford, MA). Boc amino acids were purchased from Kokusan Laboratory Chemicals, TCI, or NOVA BioChem. The biotin derivative was purchased from Pierce Biotechnology. Other chemicals were purchased from Sigma-Aldrich, TCI, or Kanto chemicals.

Synthesis of C-Man-WSPW Boc-Ser-Pro-Trp-OMe and other peptides were prepared by the conventional solution-phase Boc peptide synthesis protocol. For example, Boc-Pro-Trp-OMe (767 mg, 1.85 mmol) was dissolved in CH₂Cl₂ (20 mL), and 4.0 M HCl (20 mL, dioxane solution) was added. After the mixture had been stirred at room temperature for 2–5 h, the solvent was evaporated. Then, dioxane was added and removed *in vacuo* several times to remove HCl. The mixture was dissolved in CH₂Cl₂, then diisopropylethylamine (*i*-Pr₂NEt) (0.48 mL, 2.78 mmol), Boc-Ser-OH (570 mg, 2.78 mmol), and 1-hydroxybenzotriazole (HOBt) (375 mg, 2.78 mmol) were added. Subsequently, water soluble carbodiimide (WSCDI, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide HCl) (530 mg, 2.78 mmol) was added at 0°C. After the mixture had been stirred overnight, the solution was diluted with CHCl₃ and an aqueous 10% citric acid solution. The aqueous layer was extracted with CHCl₃. The combined layers were washed with brine and dried over Na₂SO₄. After removal of the solvent *in vacuo*, the residue was purified by silica gel column chromatography (CHCl₃:MeOH 9:1–4:1) to give the product.

The Boc group of Boc-Ser-Pro-Trp-OMe (235 mg, 0.468 mmol) was removed, as described above. Then, the residue was dissolved in dimethylformamide (DMF) (1 mL),

and *i*-Pr₂NEt (0.13 ml, 0.729 mmol), Fmoc-(C-Man)-Trp (229.5 mg, 0.39 mmol), and (7-azabenzotriazole-1-yloxy)tri-pyrrolidinophosphonium hexafluorophosphate (193 mg, 0.508 mmol) were added at 0°C (Miller et al. 2003). After the mixture had been stirred overnight, the residue was directly purified by size-exclusion chromatography [LH 20 (MeOH as eluent)] and subsequent silica gel column chromatography (CHCl₃:MeOH 9:1–4:1) to give Fmoc-(C-Man)-Trp-Ser-Pro-Trp-OMe. MS *m/z* 995 (M⁺+Na). Fmoc-(C-Man)-Trp-Ser-Pro-Trp-OMe (297 mg 0.201 mmol) was dissolved in MeOH (3 mL) and aqueous 10% NaOH (3 mL) was added. After 1 h, the mixture was purified with a SepPak cartridge (H₂O-H₂O:MeOH 4:1) to give the product. MS *m/z* 759 (M⁺+Na). (C-Man)-Trp-Ser-Pro and (C-Man)-Trp-Ser were synthesized similarly. (C-Man)-Trp-Ser-Pro; MS *m/z* 596 (M⁺+Na). (C-Man)-Trp-Ser; MS *m/z* 476 (M⁺+Na).

C-Man-WSPWC-biotin To a solution of Fmoc-(C-Man)-Trp-Ser-Pro-Trp-Cys(S^tBu)-OMe (55.6 mg, 0.0478 mmol) in MeOH (1 mL), H₂O (0.5 mL), and DMF (0.5 mL), tributylphosphine (PBu₃) (23 mL, 0.0919 mmol) was added and the mixture was stirred at room temperature for 1 h under a N₂ atmosphere. After the solvent was removed in vacuo, the residue was dissolved in DMF (0.5 mL). To the solution, (+)-biotinyl-iodoacetamidyl-3,6-dioxaoctanediamine (27 mg, 0.050 mmol) and *i*-Pr₂NEt (10 μL, 1.78 mmol) were added and the mixture was stirred overnight. The mixture was purified using LH 20 (MeOH as eluent), then silica gel column chromatography (CHCl₃:MeOH 9:1–4:1) to give the product. MS *m/z* 1512. Then Fmoc and methyl ester were removed and purified as described above. MS *m/z* 1276. The purity of C-Man-W containing peptides was determined by 400 MHz ¹H-NMR. The purity of other peptides (i.e., non-C-Man-W containing peptides) was determined by both ¹H-NMR and HPLC (Itersil ODS column, water:CH₃CN containing 0.1% TFA). The purity of all peptides was more than 95%.

Cell lines and culture RAW264.7 cells, a clonal line of mouse macrophage-like cells, were obtained from American Type Culture Collection (TIB-71). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 20% fetal calf serum (FCS) under a humidified atmosphere of 95% air and 5% CO₂ at 37°C.

Cell injury assay The viability of cultured cells was evaluated using MTT, as described (Ihara et al. 2006). The cells (1000–2000) were placed in 100 μL of medium per well in 96-well plates, and cultured overnight. After treatment with LPS for 48 h in the presence or absence of C-Man-W, C-mannosylated peptides, and peptides, 10 μL of 0.5% MTT solution was added, and the cells were incubated at 37°C for 4 h. The reaction was stopped by adding 100 μL of lysis buffer A (10% SDS and 0.1 M HCl), and then cell viability was evaluated by measuring the absorbance at 570 nm using a microplate reader. Dead cells were detected by fluorescent DNA staining using SYTOX Green (Invitrogen) (Cheung et al. 2000). Cultured cells were stained with SYTOX Green (1 μM) for 10 min, fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.2) for 30 min under darkness, and visualized by fluorescence microscopy with excitation at 485 nm and emission at 520 nm using a Carl Zeiss LSM5 microscope (Carl Zeiss, Jena, Germany), and analyzed by PASCAL analytical software.

LPS-FITC binding assay Cells were incubated at 37°C for 20 min in medium containing LPS-FITC (1 μg/mL) in the presence or absence of WSPW or C-Man-WSPW (10 μM). After a wash with PBS, the binding of LPS-FITC to the cells was analyzed using a Cytomics FC500 Flow Cytometry System (Beckman Coulter, Fullerton, CA).

Estimation of C-Man-WSPWC-biotin binding Cells were incubated at 37°C for 20 min in PBS with biotin, WSPWC-biotin, or C-Man-WSPWC-biotin (10 μM). After a wash with PBS, the cells were blocked with 3% bovine serum albumin (BSA) in PBS, incubated with FITC-conjugated Nutra-avidin (Pierce Biotechnology) for 15 min, and washed with PBS containing 1% BSA. After another wash, the binding of biotin conjugates to the cells was analyzed using a Cytomics FC500 Flow Cytometry System. Alternatively, after incubation with the biotin conjugates as described above, the cells were treated for 10 min with or without 1 mM DSP, a membrane permeable cross linker. After quenching with 1 M Tris-HCl (pH 7.2), the cells were washed with PBS, and harvested by using a scraper. The cells were lysed in lysis buffer B (20 mM Tris-HCl [pH 7.2], 130 mM NaCl, and 1% NP-40 including protease inhibitors [20 μM APMSF, 50 μM pepstatin, and 50 μM leupeptin]). Protein samples were electrophoresed on 7.5% SDS-polyacrylamide gels under non-reducing conditions and then transferred to a nitrocellulose membrane as described before (Ihara et al. 2005). The membrane was blocked with 5% skim milk in Tris-buffered saline (TBS; 10 mM Tris-HCl [pH 7.5] and 150 mM NaCl) and then incubated at 4°C for 10 min with the peroxidase-conjugated Nutra-avidin (Pierce Biotechnology) in TBS containing 0.1% Tween 20. After a wash with TBS containing 0.1% Tween 20, the blots were developed using an ECL chemiluminescence detection kit (GE Healthcare Biosciences) according to the manufacturer's instructions.

Immunoblot analysis Cultured cells were harvested and lysed in lysis buffer B, as described above. Protein samples were electrophoresed on 7.5–10% SDS-polyacrylamide gels under reducing conditions and then transferred to a nitrocellulose membrane. The membrane was blocked with 5% skim milk in TBS and then incubated at 4°C overnight with the primary antibody in TBS containing 0.1% Tween 20. The blots were coupled with the peroxidase-conjugated secondary antibodies, washed, and then developed using an ECL chemiluminescence detection kit.

JNK activity assay JNK activity was assayed using a JNK assay kit (Cell Signaling Technology) according to the manufacturer's protocol with glutathione S-transferase-c-Jun fusion protein (GST-c-Jun) as a substrate. Phosphorylation of GST-c-Jun was assessed by immunoblot analysis using specific antibody.

TNF-α assay Cells were plated in 96-well plates (10,000 cells/well), and cultured overnight. The medium was renewed. Then, cells were stimulated with LPS (1 μg/mL) in the presence or absence of C-Man-W, C-mannosylated peptides, and peptides (10 μM). After the incubation, the culture medium was collected, and the concentration of TNF-α in the sample was evaluated using a mouse TNF-α ELISA kit according to the manufacturer's instructions (BioSource International, Camarillo, CA). All experiments were done in quadruplicate.