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Lipopolysaccharide Induces Disruption of the Tight Junction via Toll-Like Receptor 4 and Tumor Necrosis Factor Alpha

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Abstract：**Background & Aims:** Inflammatory bowel disease has been suggested to be resulted from a dysregulation of the innate response system and disruption of the epithelial barrier in the gut. Our aim was to investigate the functional response of changes in the barrier function of the intestinal epithelium by LPS via TLR4.

Methods: T84 cells, a human intestinal epithelial cell line, were cultured on the Transwell filters. LDH concentration in the culture medium was measured to assess cell viability for LPS. The cells were transfected with siRNA for TLR4. LPS was added to the apical side of the cells. The barrier function of the TJ was evaluated by measuring transepithelial electrical resistance (TER). The expressions of the TLR4 in T84 cells were examined by Western blotting and Real-Time RT-PCR analysis. TNFα secretion into the medium induced by LPS was measured by ELISA.

Results: TLR4 was expressed in T84 cells, and was upregulated by LPS. TLR4 siRNA significantly suppressed the TLR4 mRNA by 50-70% ($P < 0.001$). At the LPS concentration of 10, 100, 300 μg/ml, LDH was not increased compared with control. After 24 hours, TER was decreased by 16% at LPS 10 μg/ml ($P < 0.001$), 23% at 100 μg/ml ($P < 0.001$) and 40% at 300 μg/ml ($P < 0.001$). TLR4 siRNA prevented a decrease in TER in a lower concentration of LPS (10 μg/ml) ($P < 0.01$), but not in a higher concentration. LPS induced an increase in the secretion of TNFα in a dose-dependent manner ($P < 0.01$). TLR4 siRNA could prevent the secretion of TNFα only in a lower concentration of LPS (10 μg/ml) ($P < 0.01$).

Conclusions: TLR4 siRNA attenuates LPS-induced disruption of TJ and secretion of TNFα in T84 cells. Knockdown of TLR4 may be effective to prevent an increase in permeability from LPS.

Key words：**Tight junction, Toll-like receptor4, short-interfering RNA, Lipopolysaccharide, tumor necrosis factor alpha, T84 cell line**

Introduction

In inflammatory bowel disease (IBD), chronic intestinal inflammation in response to luminal bacteria has been previously reported (1-3). Research in the pathogenesis of IBD has suggested an inappropriate mucosal immune response to luminal bacteria, flora or their products.

Intestinal epithelial cell lines constitutively express several functional Toll-like receptors (TLRs), which appear to be key regulators of the innate response system (4). In particular, Toll-like receptor 4 (TLR4), which recognizes lipopolysaccharide (LPS) of gram-negative bacteria (5), plays an important role in the innate immune response, and pathogenesis of IBD (6). The TLR4 and LPS complex activates an innate immune response, leading to the activation of transcription factor nuclear factor-kappa B (NF-κB), a key regulator of immune and inflammatory response (7, 8). In addition, the complex stimulates the release of immune and inflammatory cytokines such as tumor necrosis factor alpha (TNFα) in intestinal epithelial cell lines (9).

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On the other hand, the intestinal epithelium forms a relatively impermeable barrier between the lumen and the submucosa. This barrier function is maintained by a complex of proteins composing the tight junction (TJ) that is located at the subapical aspect of the lateral membranes (10). Disruption of the epithelial barrier in the gut has been reported to be the cause of IBD and intestinal infections (4). The disruption of the TJ has shown to be caused by proinflammatory cytokines such as TNFα and interferon gamma (IFN γ) (10). Previous reports have demonstrated that LPS disrupt TJ in intestinal epithelium and airway epithelium (11, 12). There have been several reports that permeability of IEC is regulated by knockdown of myosin light chain kinase, keratin 8, nuclear factor kappa B (NF-κB) p65 (13-15). However, the interaction of TLR4 to LPS-induced disruption of TJ has not been fully reported in IEC. In the present study, we examined whether LPS-induced disruption of TJ was associated with secretion of TNFα via TLR4 in the IEC, and that TLR4 siRNA was effective to attenuate disruption of TJ.

Materials And Methods

Cell culture

T84 epithelial cells were grown in 1/1 DMEM and Ham's F-12 medium supplemented with 15 mM HEPES (pH 7.5), 14 mM $NaHCO_3$, antibiotics, and 10% newborn cow serum. The cells were seeded on 6 well and 24 well cell culture plate (Coster, Cambridge, MA), or collagen-coated permeable polycarbonate filters with a surface area of 0.33, 4.67 cm^2 (Coster, Cambridge, MA). HT-29 epithelial cells were grown in McCoy's 5A medium with 25 mM HEPES, and 10% newborn cow serum. The cells were seeded on 6 well and 24 well cell culture plates.

LPS

LPS, from Escherichia coli serotype (Sigma chemical Co. St. Louis, MO) was diluted by FBS 5% medium to 10 μg/ml, 100 μg/ml, 200 μg/ml, 300 μg/ml, 500 μg/ml. LPS was then added to the apical side of the cells on the filters.

Westernblotting

80% confluent T84 and HT-29 cell monolayers on 4.67-cm^2 filters were washed with ice-cold phosphate-buffered saline (PBS) and then lysed in a mixture of 10 mM Tris HCl, 150 mM NaCl, 0.5% TritonX-100 including protease inhibitor cocktail tablets (complete mini; Roche Applied Science, Indianapolis). The lysate was centrifuged at 1,000 g for 30 min, and then the pellet was resuspended using a homogenizer. T84 and HT-29 whole-cell lysate proteins (25 μg per lane) were subjected to 7.5% polyacrylamide gel electrophoresis (80 V, 120 min) and then transferred (120 V, 60 min) onto a polyvinylidene difluoride membrane. The specific TLR4 proteins were detected using the different anti-TLR4 antisera (TLR4 (H-80) rabbit polyclonal IgG antibody from Santa Cruz Biotechnology, CA) (1:200) and goat anti rabbit horseradish peroxidase-conjugated secondary antibody (1:1000). Immune complexes were detected using the lumino image analyzer LAS-3000 (FUJIFILM, Tokyo).

Cell viability

LDH concentration in the culture medium was measured to assess cell viability for LPS. Confluent T84 cell monolayers on the 6 well cell culture plate were exposed to LPS (0, 10, 100, 300, 500, or 1000 μg/ml), or medium alone. Six hours later, we analyzed the LDH concentration in the culture medium.

Transfection of T84 cell with TLR4 Small interfering RNA (siRNA)

T84 cells were grown on the Transwell (6 well insert membrane growth area 4.67 cm^2, 24 well insert membrane growth area 0.33 cm^2). TLR4 siRNA was transfected into confluent T84 cells with DharmaEFCT siRNA Transfection Reagent 1 (Dharmacon RNA Technologies, Lafayette, CO). DharmaEFCT siRNA transfection reagent1 was diluted in medium without serum and incubated for 5 min. Each TLR4 siRNA (siGENOME SMART pool, Dharmacon RNA Technologies, Lafayette, CO) and negative control siRNA was diluted in medium without serum and added to the diluted DharmaEFCT siRNA Transfection Reagent 1. The mixture was incubated for 30 min at room temperature to allow the siRNA/negative control siRNA: DharmaEFCT siRNA Transfection Reagent1 complexes to form. 210 μl (25 nM) of complexes was added to each Transwell, and cells were incubated for 30-48 hours without replacement of the medium. Finally, TLR4 expression in treated cells was analyzed by real-time RT-PCR.

siGENOME SMART pool (Dharmacon RNA Technologies, Lafayette, CO) was used for TLR4 siRNA. The sequences were: CCACCUCUCUACCUUAAUA.

RNA preparation and Real-time RT-PCR analysis

T84 cells were grown on the Transwell (6 well insert membrane growth area 4.67 cm^2) for 10 days. Cells were divided into control groups and TLR4 siRNA (25 nM) transfected group. Cells were incubated for 30 hours and collected. Total RNA was isolated from T84 cells with a mirVana miRNA Isolation kit (Applied Biosystems, Foster City, CA). For RT reaction, High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) was used. The TLR4, and GAPDH oligonucleotide primers used for Ral-time RT-PCR were TaqMan® Gene Expression Assays (Applied Biosystems, Foster City, CA). TaqMan® real-time reverse transcription polymerase chain reaction assay for TLR4 was performed on an ABI prism 7500 sequence detection system according to the manufacture's protocol (Applied Biosystems, Foster City, CA).

Electrophysiological measurement of TER of cultured T-84 cells

Confluent T84 monolayers on Transwell (24 well insert membrane growth area 0.33 cm^2) were checked for transepithelial electricalresistanse (TER) before each experiment using an epithelial voltohmmeter (EVOM/EndOhm; World Precision Instruments, Sarasota, FL). Cells were used only when their stable TER was more than 1000 Ω・cm^2. The monolayers were pretreated by siRNA, or normal medium before experiment, then the monolayers exposed to the medium (5% FBS) alone or LPS (10, 100, or 300 μg/ml), added to the apical side of the cells on the filters for 24 hours. TER was measured at 6, 12, and 24 hours, after incubation with LPS.

Monoclonal Anti-human TNFα antibody

Confluent T84 monolayers on Transwell were exposed to LPS alone (0, 10, 100, or 300 μg/ml) or complex of LPS (0, 10, 100, or 300 μg/ml) and TNFα antibody (R&D Systems, Inc.) (0.05 μg/ml), added to the apical side of the cells on the filters for 6 hours. TER was measured at 6hours, after incubation with LPS or LPS and TNFα antibody.

Enzyme-linked immunosorbent assay (ELISA) for TNFα

T84 cells were grown on the 6 well and 24 well cell culture plates. T84 cells were divided into siRNA pretreated group, and normal medium group. Each group was incubated for 1 week, and added with LPS (0, 10, 100, or 300 μg/ml). 6 hours later, each medium was collected, and ELISA was performed for TNFα in the medium using Endogen Human TNFα ELISA Kit, and POWER WAVE X (Bio-TEK Instruments, VT).

Statistics

Results were expressed as means ±SD. Student's t test was used to compare the results, with statistical significances assumed at $P < 0.05$.

Results

Expressions of TLR4 proteins in T84 cells and HT-29 cells

Westernblotting demonstrated that TLR4 proteins were confirmed at the sizes of 89 kDa in both cells, and much higher amounts of TLR4 were detected in T84 cells than in HT-29 cells (Figure1). Therefore, we used T84 cell lines for the following experiments.

Cell viability

At the LPS concentration of 10, 100, 300 μg/ml, LDH concentration was not increased compared with controls (Figure 2). At the higher concentrations of 500 and 1000 μg/ml, LDH concentration was increased ($P < 0.001$). These results suggested that cells were not disrupted up to the concentration of 300 μg/ml of LPS.

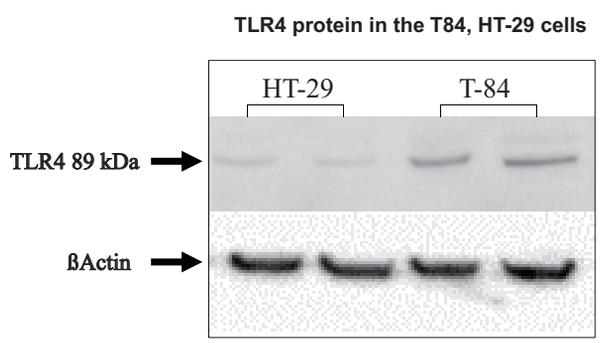

Figure 1. Immunoblotting for TLR4. Confluent T84, and HT29 intestinal monolyer cells were incubated in normal medium without LPS. The specific TLR4 proteins were detected using the different anti-TLR antisera (TLR4 (H-80) rabbit polyclonal IgG antibody). TLR4 proteins were confirmed at the size of 89 kDa in both cells, but much lower amounts of TLR4 were detected in HT-29 than in T84.

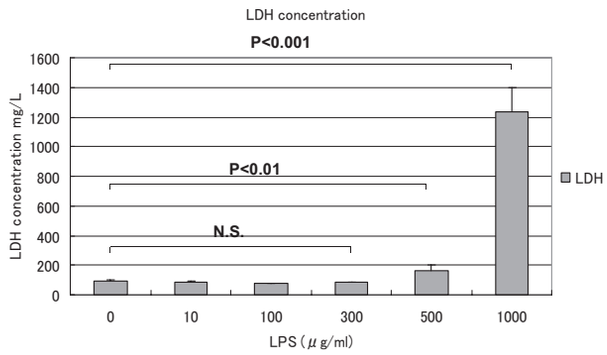

Figure 2. LDH in the culture medium was analyzed to assess T84 cell viability for LPS. At the LPS concentration of 10, 100, 300 μg/ml, LDH was not increased compared with controls. At the high concentration of 500 and 1000 μg/ml, LDH was increased ($P < 0.001$). Results represent the mean ± SD (n = 5)

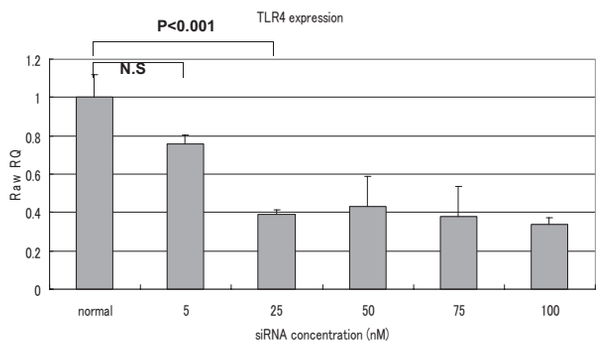

Figure 3. TLR4 siRNA was transfected into confluent T84 cells on the Transwell. The expression of TLR4 mRNA was analyzed by real-time RT-PCR. The TLR4 siRNA significantly suppressed the TLR4 mRNA expression by 50-70% ($P < 0.001$) in siRNA concentration more than 25 nM. Results represent the mean ± SD (n = 4)

siRNA decreases TLR4 mRNA

TLR4 siRNA significantly suppressed the TLR4 mRNA expression by 50-70% ($P < 0.001$) at the siRNA concentration more than 25 nM (Figure 3). Therefore, we used TLR4 siRNA in concentration 25 nM for experiments. The most effective period for TLR4 mRNA suppression was 30-48 hours (Data not shown).

LPS increases paracellular permeability in T84 monolayers

LPS induced a significant decrease in TER in a dose-dependent manner in control group ($P < 0.001$) (Figure 4). TER was decreased after 24 hours by 16% at LPS

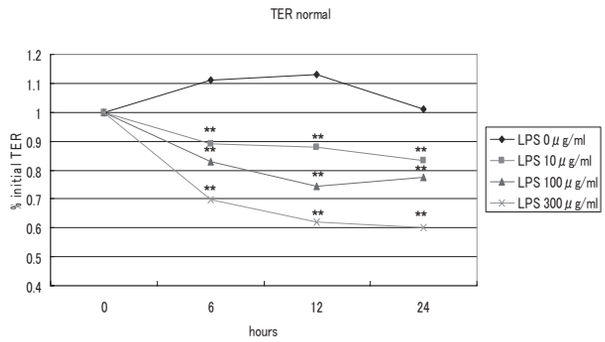

Figure 4. Electorophysiological measurement of TER of cultured T84 cells. Confluent T84 intestinal monolayers were incubated with LPS (0, 10, 100, or 300 μg/ml) added to the apical side of the monolayers or medium alone. LPS induced a significant dose-dependent decrease in TER in the T84 monolayer (*$P < 0.05$; **$P < 0.001$ compared to the control group). Results represent the mean ± SD (n = 5).

10 μg/ml ($P < 0.001$), 23% at 100 μg/ml ($P < 0.001$) and 40% at 300 μg/ml ($P < 0.001$), respectively. TER was also determined with TLR4 siRNA (siRNA group) compared with control medium (control group) at each concentration of LPS (0, 10, 100, or 300 μg/ml) (Figures 5A, B, C, D). TLR4 siRNA significantly attenuated a decrease in TER at LPS 10 μg/ml, but not at LPS (0, 100, 300 μg/ml) ($P < 0.01$). These results showed the suppression of TLR4 by siRNA attenuated LPS-induced alterations in paracellular permeability in a lower concentration of LPS (10 μg/ml).

Lower concentration of LPS-induced TNFα secretion is suppressed by siRNA in T84 cells

Cultured T84 cells were divided into siRNA group and control group. In each group, cultured cells were treated with or without LPS (10, 100, 300 μg/ml). Six hours later, we analyzed the concentration of TNFα in culture medium by ELISA. In control group, LPS induced an increase in TNF secretion in a dose-dependent manner ($P< 0.01$) (Figure 6). In the siRNA group, TNFα secretion was suppressed by 50% ($P< 0.01$) compared with control group in a low concentration of LPS (10 μg/ml) (Figure 7). These results showed that LPS induced a secretion of TNFα in T84 cells in a dose-dependent mannner, and TLR4 siRNA could suppress the secretion of TNFα in a lower concentration of LPS (10 μg/ml).

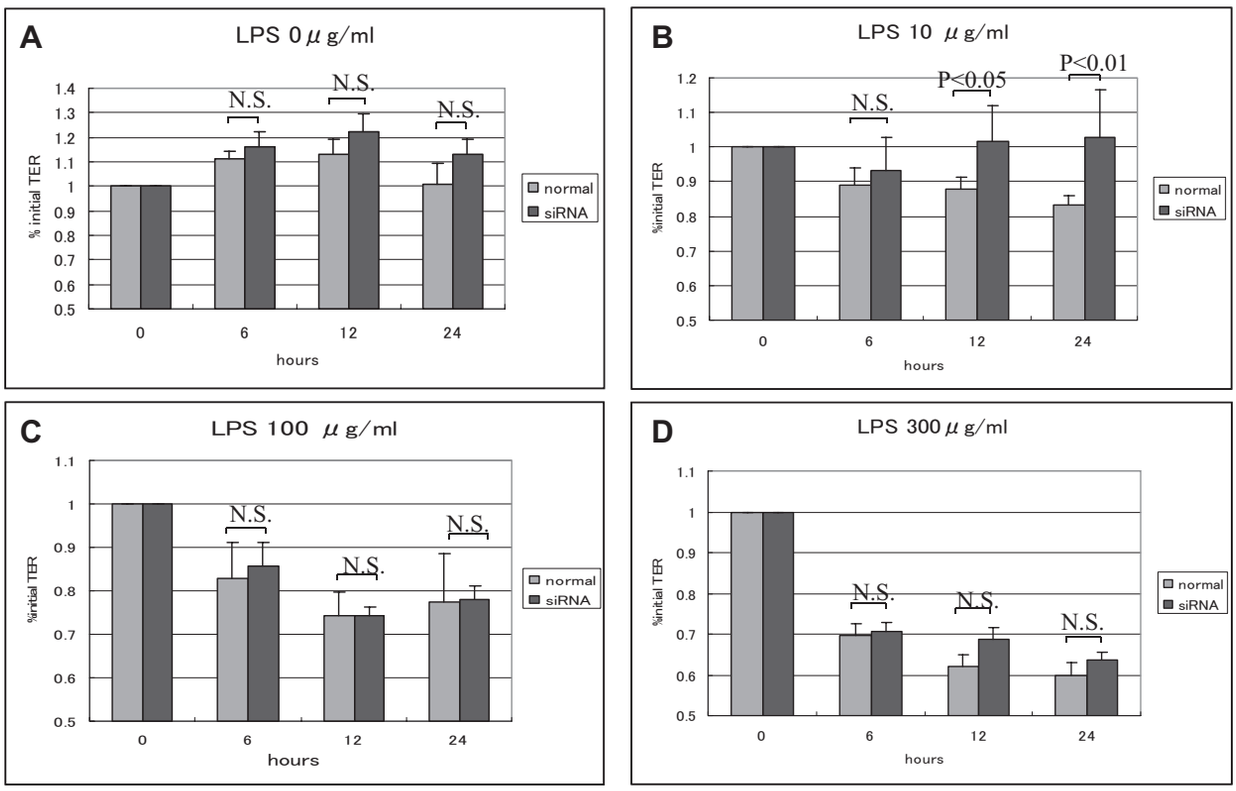

Figure 5. TLR4 siRNA was transfected in cultured T84 cells (siRNA group). TER was also determined with TLR4 siRNA (siRNA group) compared with control medium (control group) at each concentration of LPS (0, 10, 100, or 300 μg/ml) (Figures 5A, B, C, D). TLR4 siRNA significantly attenuated a decrease in TER at LPS 10 μg/ml (Figure 5B) ($P < 0.01$), but not at LPS (0, 100, 300 μg/ml) (Figure 5A, C, D). Results represent the mean ± SD (n = 5).

Monoclonal Anti-human TNFα antibody neutralizes TNFα bioactivity

Cultured T84 cells were treated with LPS (0, 10, 100, 300 μg/ml) or comprex of LPS (0, 10, 100, 300 μg/ml) and anti-human TNFα antibody (0.05 μg/ml). Six hours later, we analyzed the TER. In control group, LPS induced an increase in TNFα secretion in a dose-dependent manner ($P< 0.01$) (Figure 6). In the siRNA group, TNFα secretion was suppressed by 50% ($P< 0.01$) compared with control group in a low concentration of LPS (10 μg/ml) (Figure 7). These results showed that LPS induced a secretion of TNFα in T84 cells in a dose-dependent mannner, and TLR4 siRNA could suppress the secretion of TNFα in a lower concentration of LPS (10 μg/ml).

TLR4 upregulation by LPS

Cultured T84 cells were incubated with LPS (10, 100, or 300 μg/ml) or medium alone, the expression of TLR4 mRNA increased by 157% ($P < 0.01$) in a high concentration of LPS (300 μg/ml) (Figure 8). It is suggested that LPS upregulated TLR4 mRNA .

Discussion

In the present study, we have verified that LPS-induced disruption of TJ is mediated through TLR4 and TNFα by

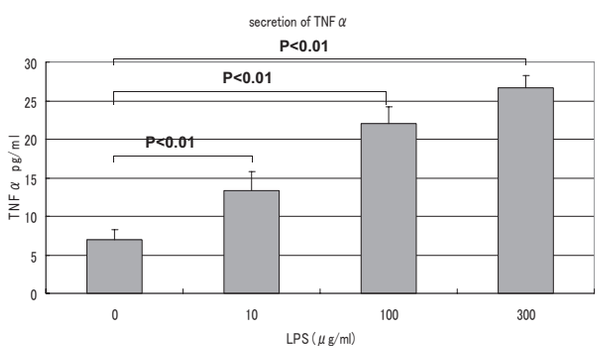

Figure 6. Confluent T84 intestinal monolayers were incubated with LPS (0, 10, 100, or 300 μg/ml) added to the apical side of the monolayers. 6 hours later, we analyzed the concentration of TNFα in the medium by ELISA. LPS induced a increase of TNFα in a dose-dependent manner ($P < 0.01$). Results represent the mean ± SD (n = 4).

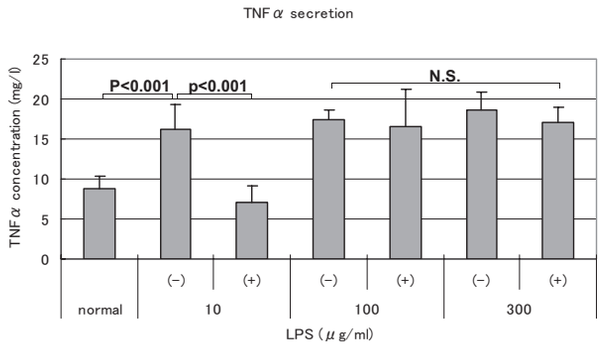

Figure 7. TLR4 siRNA was transfected into T84 intestinal monolayers. Cells were incubated with LPS (0, 10, 100, or 300 μg/ml) added to the apical side of the monolayers. 6 hours later, we analyzed the concentration of TNFα in the medium by ELISA. TNFα secretion was suppressed by 50% compared with control group in the LPS 10 μg/ml ($P < 0.001$). Results represent the mean ± SD (n = 4).

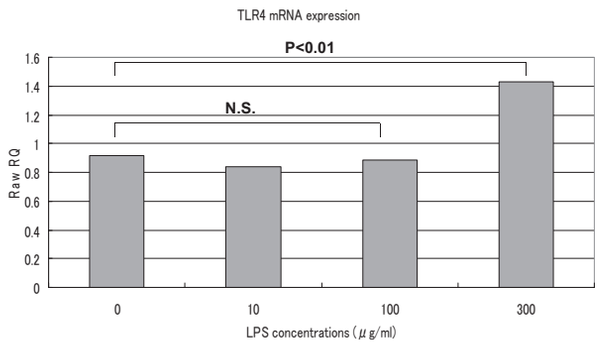

Figure 8. Cultured T84 cells were incubated with LPS (10, 100, or 300μg/ml) or medium alone, the expression of TLR4 mRNA was analyzed Real-time RT-PCR. The TLR4 mRNA increased by 157% ($P < 0.01$) in the high concentration of LPS (300μg/ml). Results represent the mean ± SD (n = 4).

using TLR4 siRNA.

IBD, including Crohn's disease (CD) and ulcerative colitis (UC), is an immune-mediated illness that is characterized by chronic intestinal inflammation, and their etiologies are as yet unknown (16). One theory of pathogenesis suggests that an immune imbalance might result from an exaggerated activation of the mucosal innate immune system in response to the bacterial products of the lumen and disruption of the epithelial barrier in the gut (4). The intestinal epithelium forms a relatively impermeable barrier between the lumen and the submucosa. This barrier function is maintained by a complex of proteins composing the TJ that is located in the paracellular space between epithelial cells and between endothelial cells (10). Its regulates the paracellular permeability of these cell layers (16).

The disruption of the TJ has been reported to be caused by proinflammatory cytokines such as TNFα, IFN-γ (16, 17, 28) and interleukin-1 (19). Bannerman et al. reported that staphylococcus enterotoxin B induced a loss of barrier function in endothelial cells (20). LPS increases paracellular permeability in intestinal epithelial cells, cholangiocytes and the airway epithelium (12, 21, 22). In the present study, LPS induced a decrease in TER in a dose-dependent manner. It indicates that LPS disrupts the TJ in an intestinal epithelial cell line. Youakim *et al*. reported that disruption of the epithelial barrier in the gut is an inducement of inflammatory bowel disease and intestinal infections, suggesting that LPS has an important role in the pathogenesis of chronic intestinal inflammation (10).

It is well known that TNFα is secreted by monocytes, macrophages and neutorophila following their stimulation by bacterial LPS (23). Previous reports and our present study indicated that T84 cells produce TNFα (24). Furthermore, in the present study, LPS induced an increase in secretion of TNFα in a dose-dependent manner. Particularly, it has been also demonstrated that TNFα-induced increase in intestinal epithelial TJ permeability is mediated downregulation of zonura occludens (ZO)-1 protein, a protein of TJ, by NF-κB (27), and myosin regulatory light chain kinase (11). In our study, LPS decreases TER in a dose-dependent manner. This suggests that LPS-induced disruption of TJ is mediated by TNFα secretion. Taylor *et al*. reported that T84 cells produced TNFα in hypoxia condition, and the IFN γ, a cytokine that is increased in the intestinal mucosa during inflammation, upregulates cell surface TNFα receptors. These findings indicate a functional correlation in the regulation of epithelial permeability through autocrine pathways. Similarly, our report demonstrated that LPS-induced secretion of TNFα disrupts TJ through autocrine pathways.

Cario *et al*. reported that intestinal epithelial cell lines constitutively expressed several functional Toll-like receptors (TLRs) (4). TLRs are members of the pattern recognition receptor family and are characterized by an extracellular domain with leucine-rich repeats and an intracellular domain homologous to the Toll/IL-1R (26, 27). To date, there have been 13 identified mammalian TLRs, 11 human TLRs and 13 mouse TLRs (28). In

particular TLR4 recognizes LPS (29, 30), and plays an important role in the innate immune response. The TLR4 and LPS complex activates transcription factor NF-κB, and releases proinflammatory cytokines such as TNFα (7, 8), IFN γ (10, 16, 17). In the present study, TLR4 was detected in human intestinal epithelial cell lines (T84 and HT-29). Luminal bacteria can produce a vast variety of toxic and proinflammatory constituents. LPS, a glycolipid derived from outermost membrane of gram-negative bacteria in the gut, is one of the most abundant at the apical IEC surface (31). In the present study, LPS induced an increase in secretion of TNFα in a dose-dependent manner. Thus, it is suggested that LPS-induced disruption of TJ is mediated by TNFα secretion via TLR4 in the IEC.

We hypothesized that suppression of TLR4 prevents the secretion of TNFα and disruption of TJ. Then, we examined whether knockdown of TLR4 could decrease LPS-induced disruption of TJ in the IEC by using TLR4 siRNA. In the present study, TLR4 siRNA suppressed TLR4 mRNA by 50-70% in T84 cells. Furthermore TLR4 siRNA prevented LPS-induced decrease of TER in a low concentration of LPS (10 μg/ml). These results suggest that TLR4 has an important role on the disruption of TJ. Indeed, there have been several reports demonstrating that TLR4 was strongly upregulated in both UC and CD (4). Fukata *et al*. reported that DSS treatment of TLR4-/- mice was associated with striking reduction in acute inflammatory cells compared with wild-type mice (5). Similarly our data showed that suppression of TLR4 in the IEC is effective for prevention of LPS-induced TJ disruption. On the other hand, Fort *et al*. have shown that CRX-526, which has antagonistic activity for TLR4, can block the interaction of LPS with immune system, and then blocks of LPS to induce TNFα release. They have clearly demonstrated that CRX-526 could block the development of moderate-to-sever colitis in mouse models of chronic inflammation (32). Thus, the blockage of TLR4 is considered to be effective to reduce an LPS-induced TNFα secretion.

The concentration of TNFα has been shown to be increased in serum and the tissues of patients with IBD (16). Infliximab, which is a genetically constructed IgG1 murine-human chimeric molecule that binds both soluble and membrane bound TNFα, is effective for IBD. Grabig *et al*. reported that antibiotic as well as probiotic therapy attenuates both experimental colitis and human IBD. (26). Both TNFα and luminal content such as bacteria are considered to be important in the pathogenesis of chronic intestinal inflammation.

In summary, our present study suggests that LPS-induced disruption of TJ was associated with secretion of TNFα via TLR4 in the IEC, and TLR4 siRNA was effective to prevent disruption of TJ. In addition to anti-TNFα antibody and antibiotics, the inhibition of TLR4 could be a promising candidate in treatment of IBD.

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