Cellular differentiation-induced attenuation of LPS response in HT-29 cells is related with down-regulation of TLR4 expression

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I am dedicating this thesis to my parents, my sons who

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Sang Kil Lee

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Abstract

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Intestinal epithelial cells are not only a physical barrier to bacteria but also display active role in immune and inflammatory responses. The migration of epithelial cells from the crypt base to the surface is accompanied by cellular differentiation that leads to important morphological and functional changes. It has been reported that differentiation of intestinal epithelial cells is associated with reduction of interleukin (IL)-8 response to IL-1β . Although toll-like receptors4 (TLR4) has been identified as one of the important components of innate immunity to lipopolysaccaride (LPS) in intestine, little is known about regulation of TLR4 in intestinal epithelial cells during cellular differentiation. We investigated the effects of differentiation on LPS-induced IL-8 secretion and expression of TLR4. Differentiation was induced by treatment with butyrate or post-confluence culture and was assessed by measuring alkaline phosphatase (AP) activity. IL-8 secretion was measured by ELISA. TLR4 protein and mRNA expressions were examined by Western blot and RT-PCR, respectively. Immunohistochemistry

as performed to demonstrate spatial expression of TLR4 along the crypt-villus axis in human small intestine.

HT-29 cells were dose-dependently responsive to LPS. AP activity was increased in HT-29 cells by differentiation induced by treatment with butyrate or post-confluence culture. IL-8 secretion induced by LPS was strongly attenuated in differentiated cells compared with that in undifferentiated cells. Cellular differentiation also attenuated TLR4 mRNA and protein expressions. Pretreatment with tumor necrosis factor (TNF)-α and interferon (INF)-γ in HT-29 cells augmented not only LPS-induced IL-8 secretion but also TLR4 expression. These TNF-α - or INF-γ -induced augmentations of LPS response as well as TLR4 expression were down-regulated by differentiation. In human small intestine, TRL4 was expressed predominantly in epithelial cells located in the lower portion of the crypts.

Collectively, we concluded that cellular differentiation attenuated IL-8 secretion induced by LPS in HT-29 cells and this attenuation was related with down-regulation of TLR4 expression.

Key Words: differentiation, IL-8, LPS, TLR4, butyrate.

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<Directed by Professor **Won Ho Kim**>

I. Introduction

The crypt-villus axis of the intestinal mucosa is composed of a dynamic cell population that migrates from a proliferative, undifferentiated cells in crypt to mature, differentiated surface epithelial cells. Although several studies have shown that migration of epithelial cells from the crypt base to surface accompanied by cellular differentiation which involves substantial changes of cellular morphology, growth, proliferation, and expression of biochemical markers,[1, 2] little is known about the alteration of immuno-inflammatory functions during epithelial differentiation. In recent years, it has become clear that the intestinal epithelium also serves as the defensive frontline of the mucosal innate immune system in the gastrointestinal tract.[3, 4] Despite the intestinal epithelium on the mucosal surface is continually exposed to a high intraluminal concentration of diverse bacteria and their products,[5, 6] the intestinal mucosa maintains a controlled state of inflammation. By contrast, invasive and toxin-producing pathogenic bacteria elicit acute inflammation and secretion of pro-inflammatory cytokines by intestinal epithelial cells. In this point, controlled immune response of epithelial cell may be regarded as a main mechanism

of self-defense.

During chronic intestinal inflammatory states, however, there is an alteration in this pattern of epithelial differentiation companied by morphological changes. The increase in epithelial proliferation with an expansion of cell populations in an undifferentiated state causes crypt hypertrophy, while a decrease in cells exhibiting a differentiated phenotype conduces villus atrophy.[7] Crypt hypertrophy and relative undifferentiation of cells in the mid-crypt region are common features observed in the actively inflamed gut from patients with inflammatory bowel disease.[8] The molecular mechanisms responsible for alteration of epithelial differentiation during intestinal inflammatory states are currently unknown.

Indirect evidences suggest that the state of epithelial cellular differentiation plays a role in the immune responsiveness of the intestinal mucosa. For example, only undifferentiated proliferating rat colonic crypt epithelial cells are capable of producing the pro-inflammatory cytokine, IL-1β .[9] Furthermore, the enteroinvasive bacteria *Yersinia pseudotuberculosis* which can invade undifferentiated Caco-2 cells is unable to infect differentiated cells due to the redistribution of a bacterial cell receptor during the process of cellular differentiation.[10] Recently, it has been reported that cellular differentiation of HT-29 cells impair the IL-1β signaling pathways including both nuclear factor (NF)-κ B and c-Jun N-terminal kinase (JNK).[11]

Short chain fatty acids (SCFA) are normal products of anaerobic bacterial fermentation of carbohydrates in the colon and are the major source of nutrition for the human colonic epithelium. Butyrate, the SCFA most avidly metabolized by colonocytes, can induce markers of a differentiated phenotype in multiple cell types[12] and is demonstrated to be an effective

treatment for distal ulcerative colitis.[13-16] Evidences have also been obtained that butyrate can influence cytokine-activated gene expression in colonic epithelial cells.[17, 18] The mechanism of anti-inflammatory effect of butyrate is supposed to be related with suppression of NF-κ B activity.[19, 20]

LPS, glycolipid of the cell wall of Gram-negative bacteria, stimulates intestinal epithelial cell to secrete IL-8, a chemotactic and activating peptide for neutrophils.[21, 22] Recently Toll-like receptors (TLRs) have been identified as important components of innate immunity.[23, 24] Ten TLRs have been cloned in humans, but only TLR2 and TLR4, transfected into cells devoid of TLRs, have been shown to provide the transmembrane domain in a CD14/TLR complex, required to transmit LPS signals by induction of NF-κ B.[25, 26] Regulation of these receptors and their downstream pathways represents a possible pathway to modify LPS-induced immune responses, exemplified by the C3H/HeJ mouse with hypo-responsiveness to LPS due to a mutation of the *lps*-locus encoding for TLR4.[27] Furthermore, TLRs expression in intestinal epithelial cell is altered in inflammatory bowel disease, for example, TLR4 is up-regulated both in Crohn's disease and ulcerative colitis,[28] and TLR4 and MD-2 were shown to synergically activate the transcriptional factor NF-κ B in intestinal epithelial cell.[29] So far little is known about changes in TLR4 expression and their function during cellular differentiation in epithelial cells. Therefore, we determined the influence of differentiation on LPS-induced IL-8 secretion in conjunction with TLR4 expression in colonic epithelial cells.

II. Materials and Methods

1. Cell culture and treatment

The human colonic epithelial cells HT-29 (American Type Culture Collection, Rockville, MD, USA; ATCC HTB 38), Caco-2 (ATCC HTB37) and human monocytic cell line THP-1 (ATCC TIB202) were used between passages 20-40 and grown in RPMI-1640 medium (Gibco-BRL, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco-BRL), containing 100 IU/ml penicillin and 100 μg/ml streptomycin. THP-1 cells, differentiated by treatment with 10 ng/ml of phorbol myristate acetate (Sigma Chemical Co, St. Louis, MO, USA) for 18 h, were used as a positive control for TLR4 expression.[30]

Experiments were performed with subconfluent cells representing undifferentiated status and differentiated cells induced by treatment with sodium butyrate (Sigma Chemical Co.) to a final concentration of 1-3 mM for 48 h or post-confluence culture up to 7 days with changes of medium every 2 days. Measurement of AP activity was performed as described elsewhere.[31]

The following bacterial antigens, metabolites and cytokines were used: LPS (10 ng/ml-1 μg/ml; *Escherichia coli* strain B8:0127; Sigma Chemical Co.), TNF-α (10 ng/ml and 20 ng/ml; R&D Systems, Minneapolis, MN, USA), IL-1β (0.1 ng/ml; R&D Systems), and INF-ɣ (40 ng/ml; R&D Systems).

The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide] assay was used to quantify cell viability.

2. IL-8 Enzyme-linked immunosorbent assay (ELISA)

Monoclonal anti-IL-8 antibody (R&D Systems) suspended

in the coating buffer (14.2 mM Na2CO3, 34.9 mM NaHCO3, 3.1 mM NaN3, pH 9.6) was added to a microtiter plates (Costar, Corning, NY, USA), and incubated overnight at 4℃. The plate was washed three times with PBST (0.05% Tween-20), and blocked with PBST containing 1% bovine serum albumin (BSA) for 1 h at 37℃. One hundred μl of sample or diluted standard IL-8 (R&D Systems) was added to the plate, and incubated for 2 h at room temperature. After washing with PBST, 100 μl of biotinylated goat anti-mouse IgG (R&D Systems) was added to the plate and incubated for 1 h at 37℃, followed by incubation with streptavidin-horseradish peroxidase (HRP) conjugate (Amersham, Buckinghamshire, UK). Color was developed by adding 100 μl of Tetramethylbenzidine (Sigma Chemical Co.) to the plate. After 30 min of incubation at room temperature, the reaction was stopped with 4 M H_2SO_4 and the OD value was measured at 450 nm using an ELISA reader (Gibco-BRL).

3. Western blotting

The expression of TLR4 was determined by Western blotting. HT-29 cell lysates were prepared using an ice cold lysis buffer (50 mM Tris, pH 7.4, 1 mM EDTA, 0.1% Triton X-100, 1 mM PMSF, 25 μg/ml leupeptin, 20 μg/ml pepstatin). Equivalent protein samples were resolved on 10% SDS-polyacrylamide gels and transferred to polyvinylidenedifloride (PVDF) membrane (Millipore, Bedford, MA, USA). The membrane was blocked with TBST containing 5% non-fat milk for 30 min at room temperature. For immunodetection, the membranes were incubated overnight with anti-TLR4 antibody (1:200, Santa Cruz Biotechnologies Inc, Santa Cruz, CA, USA) in Tris buffered saline/Tween 20 with 1% milk powder, followed by incubation with the HRP conjugated anti rabbit-IgG (1:5000,

Amersham). The bands were visualized by enhanced chemiluminescence (ECL-kit, Amersham).

4. RT-PCR

mRNA was isolated from HT-29 cells using TRIzol method (Invitrogen, Carlsbad, CA, USA). Integrity and purity of isolated RNA were assessed by electrophoresis on a 1.2% agarose gel before generation of cDNA using reverse transcription. For reverse transcription, a cDNA reaction mixture was made using 1 μg of RNA in a 20 μl reaction mixture containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM dNTP, and 200 units of SuperScrip II reverse transcriptase (Invitrogen). PCR reactions were set up in a mixture of 20 μl containing 1 μg template, 2.5 mM each dNTP, 1 X PCR buffer, 10 pmol primer, and 1.5 units of Ex Taq ^{TM} Polymerase (TaKaRa BIO Inc, Shiga, Japan). Amplification was used the following conditions: initial denaturation: 95℃ for 5min, followed by 30 cycles of denaturation at 92℃ for 45 sec, annealing at 56℃ for 45 sec, and elongation at 72℃ for 2 min. PCR products were separated in 1.5% agarose gel. The oligonucleotide primers used for RT-PCR were as follows: human TLR4: 5'-TGTCCC TGAACCCTATGAAC-3' sense, 5'-GCCTTTTGAGAGATTTGAGT-3' anti-sense. β-actin: 5'-CCAACCGCGAGAAGATGACC-3' sense, 5´-GATCTTCATGAGG TAGTCAGT-3' anti-sense.[29]

5. Immunohistochemistry

Normal intestinal tissues of human small and large bowel were obtained form patients who undertook right hemicolectomy for ascending colon cancer. A standard avidin-biotin-immunoperoxidase technique was performed. Briefly, 4 μm tissue

sections were dewaxed and rehydrated through graduated changes of xylene and graded alcohol, then to water. Endogenous peroxidase activity was blocked by incubating the sections with 0.6% hydrogen peroxide for 10 min. Heat mediated antigen retrieval was performed by heating the sections (immersed in 0.01 M citrate buffer, pH 6.0) in a microwave oven (750 W) for 20 min. The slides were then washed with PBS before being exposed to 10% normal bovine serum for 10 min to block non-specific background reactions. The slides were then incubated with anti-TLR4 antibody (1:200, Santa Cruz Biotechnologies Inc.) overnight at 4 ℃. Following washes with PBS, the slides were incubated for 15 min with universal secondary antibody (DAKO Lab, Carpinteria, CA, USA), further washed for 3 × 10 min in PBS, and incubated with peroxidase-conjugated streptavidin (Sigma) for 10 min. The peroxidase reaction was developed in PBS using hydrogen peroxide as substrate and DAB as a chromogen, and sections were counterstained with haematoxylin, dehydrated, and evaluated under a light microscope. For negative control, secondary antibody was not added.

6. Statistical analysis

The statistical software program used was the Statistical Package for the Social Sciences (SPSS/PC+ 10.0, Chicago, IL, USA). Significance was accepted at a p value of less than 0.05.

III. Results

1. Colon cancer cell lines, HT-29 and Caco-2 cells, differentially respond to LPS

HT-29 and Caco-2 cells were cultivated until 70% confluence and then stimulated by IL-1β (0.1 ng/ml), TNF-α (1 ng/ml) and LPS (100 ng/ml) for 12 h prior to assessment of cell-free supernatant for IL-8 content. In Caco-2 cells, IL-1β and TNF-α induced IL-8 secretion and its level was elevated to about 11.3- and 6.9- folds of control level, respectively, while Caco-2 cells were unresponsive to LPS (Fig. 1A). In contrast, not only IL-1β and TNF-α but also LPS increased IL-8 secretion in HT-29 cells and its level was elevated to about 6.6-, 7.2- and 7.1- folds of control level, respectively. Western blot demonstrated expression of TLR4 in HT-29 but not in Caco-2 cells (Fig. 1B).

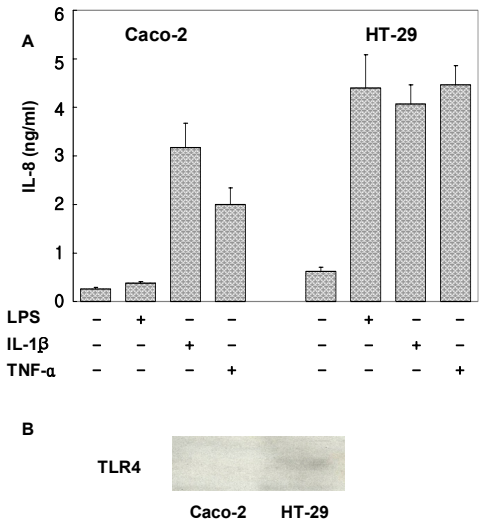

Figure 1. LPS response and TLR4 expressions in Caco-2 and HT-29 cells. (A) Caco-2 and HT-29 cells were stimulated with LPS (100 ng/ml), IL-1β (1 ng/ml) or TNF-α (10 ng/ml) for 12 h. IL-8 in the supernatant was measured

using an ELISA technique. These results are expressed as means of triplicate determinations and are representative of three independent experiments. (B) Constitutive TLR4 protein expressions in Caco-2 and HT-29 cells were analyzed by Western blot.

LPS dose-dependently induced secretion of IL-8 in HT-29 cells reaching a plateau level at 100 ng/ml concentration (Fig. 2). These results verifies close relationship between TLR4 expression and LPS response, recently demonstrated by acquirement of LPS responsiveness after transfection with both TLR4 and MD-2 cDNA.[32]

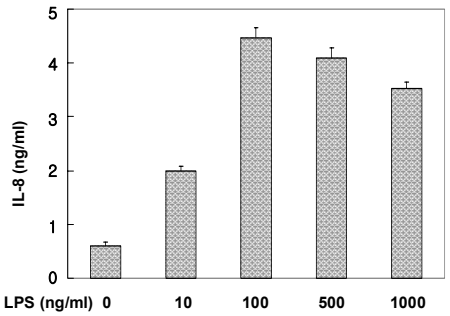

Figure 2. Dose-dependent induction of IL-8 production by LPS. HT-29 cells were treated with 0-1000 ng/ml of LPS and cultivated for 12 h prior to assessment of cell-free supernatant for IL-8 secretion by ELISA.

2. AP activities are induced in HT-29 cells during spontaneous and butyrate-induced differentiation

AP has been well characterized as a marker of cell differentiation, increasing spontaneously in Caco-2 cells with time in culture, and in numerous cell lines, including HT-29 cells, after butyrate treatment.[33-35]

Treatment of sub-confluent HT-29 cells with butyrate increased AP activity in a dose-dependent fashion. Butyrate at 3 mM increased AP activity by 700% of control (Fig. 3A). AP activity was also increased by 325% of control (Fig. 3B) at 7 day post-confluence culture. These results demonstrate that not only butyrate but also post-confluent culture induces differentiation in HT-29 cells.

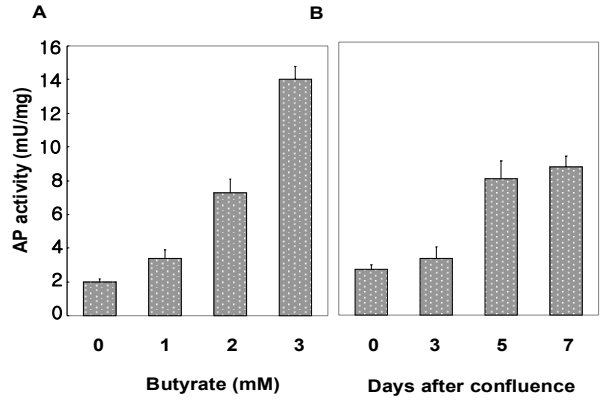

Figure 3. Increased AP activity by differentiation. (A) Subconfluent HT-29 cells were treated with butyrate (0-3 mM) for 48 h. (B) After confluence, HT-29 cells were cultured for up to 7 days with changes of medium every 2 days. Then supernatants were harvested for measurement of IL-8.

3. Differentiation attenuates LPS-induced IL-8 secretion in HT-29 cells

We then investigated whether differentiation of HT-29 cells would lead to alteration of LPS responsiveness. HT-29 cells differentiated by butyrate treatment (0-3 mM) or post-confluence culture (0-7 days) were stimulated with LPS (100 ng/ml) and IL-8 secretion was measured by ELISA. Butyrate pretreatment completely abolished LPS-induced IL-8 secretion (Fig. 4A). Furthermore, LPS-induced IL-8 secretion was also

strongly inhibited by post-confluence culture (Fig. 4B). These results show that cellular differentiation in epithelial cells attenuates IL-8 response to LPS.

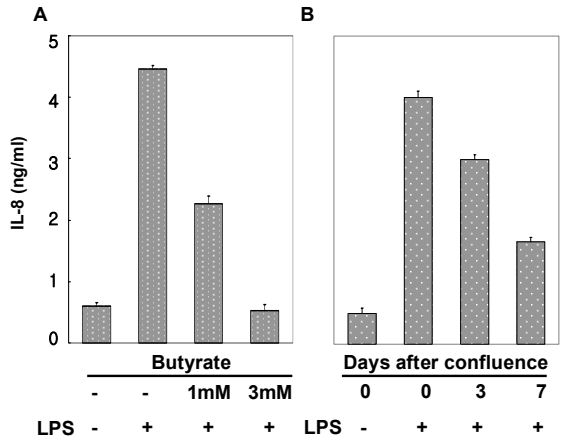

Figure 4. Attenuation of LPS-induced IL-8 secretion by differentiation. (A) Sub-confluent HT-29 cells were pretreated with butyrate (0-3 mM) for 48 h, then were stimulated with LPS (100 ng/ml) for 12 h. (B) After confluence, HT-29 cells were cultured for up to 7 days with change of medium every 2 days, then were stimulated with LPS (100 ng/ml) for 12h. Then supernatants were harvested for measurement of IL-8.

4. Differentiation also attenuates TLR4 expression in HT-29 cells

Since TLR4 has been identified as important components of innate immunity and particularly LPS responsiveness,[32, 36] we determined constitutive expression of TLR4. TLR4 mRNA and protein were detectable in HT-29 cells at sub-confluence state, while treatment of HT-29 cells with butyrate (3 mM for 48 h) reduced TLR4 mRNA and protein levels by 67% and 25%, respectively (Fig. 5A and 5C).

Furthermore, as compared to TLR4 mRNA and protein of sub-confluent HT-29 cells, those in HT-29 cells cultured 7 days after confluence were reduced by 25% and 22%, respectively (Fig. 5B and 5D). These results suggest that cellular differentiation in colonic epithelial cells is associated with down-regulation of TLR4.

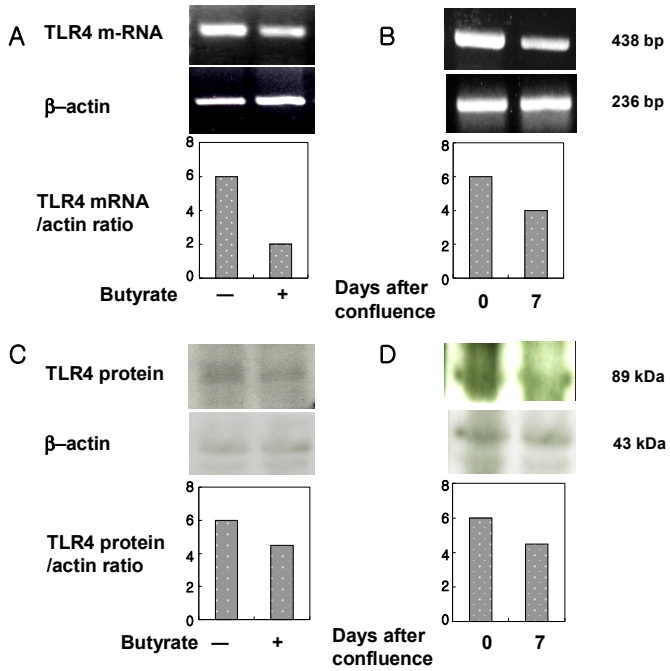

Figure 5. Attenuation of TLR4 expression by butyrate-induced (A, C) and spontaneous differentiation (B, D). RT-PCR was performed with specific primers of indicated receptor components for TLR4 and β - actin in (A) and (B). TLR4 protein was assessed by Western blot analysis in (C) and (D).

5. Differentiation attenuates cytokine-induced augmentation of LPS response

IFN-ɣ has recently been shown to stimulate TLR4

expression in various cells, including endothelial cells, HL-60 monocytic cells and HT-29 cells.[32, 36, 37] We investigated the effects of differentiation on TNF-α - and INF-ɣ -induced augmentation of LPS response in HT-29 cells.

Pre-incubation of HT-29 cells with TNF-α (20 ng/ml) or INF-ɣ (40 ng/ml) followed by LPS (100 ng/ml) increased IL-8 production 1.54- and 1.59-folds, respectively, compared with secretion of IL-8 in response to LPS alone (Fig. 6). The effect of differentiation on TNF-α - and INF-ɣ -induced augmentation of LPS response was also evaluated. Butyrate-induced differentiation attenuated TNF-α - and INF-ɣ -induced augmentation of LPS response by 68.8% and 69.6%, respectively (Fig. 6A). In addition, seven days of post-confluence culture attenuated TNF-α - or INF-ɣ -induced augmentation of LPS response by 41.3% and 32.2%, respectively (Fig. 6B). These results demonstrate that differentiation attenuates not only LPS response but also cytokine-induced augmentation of LPS response.

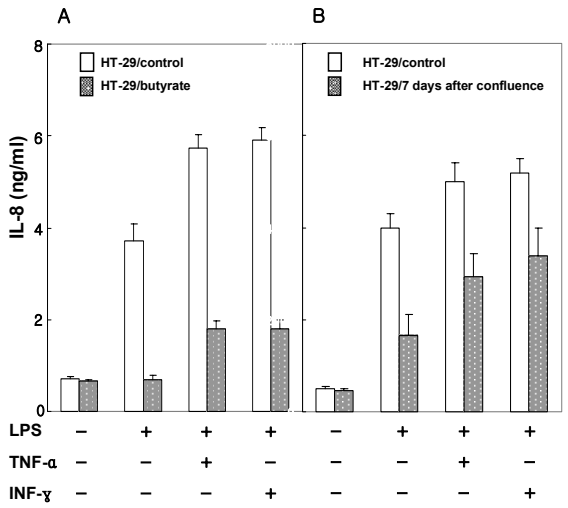

Figure 6. Attenuation of cytokine-induced enhancement of LPS response by butyrate-induced (A) and spontaneous differentiation (B). Subconfluent and

differentiated HT-29 cells were stimulated by LPS (100 ng/ml) with or without pre-exposure to 20ng/ml TNF-α or 40ng/ml INF-ɣ for 12 h. Supernatants were harvested for measurement of IL-8.

6. Differentiation also attenuates cytokine-induced up-regulation of TLR4 expression

We next wished to test the effect of differentiation on TNF-α - and INF-ɣ -regulated TLR4 expression. TNF-α and INF-ɣ increased expression of TLR4 mRNA in undifferentiated HT-29 cells. Pre-incubation with butyrate (3 mM) almost completely attenuated this increase (Fig. 7A). Furthermore, post-confluence culture also attenuated TLR4 up-regulation induced by TNF-α and INF-ɣ (Fig. 7B). These results reinforce relationship between differentiation-induced attenuation of LPS response and down-regulation of TLR4 expression.

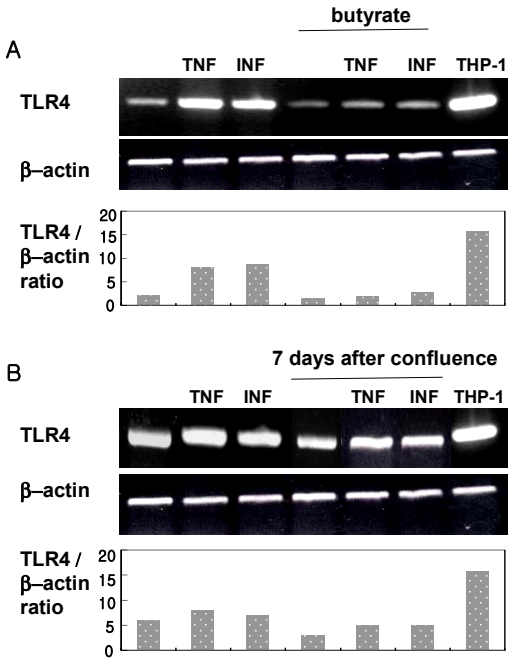

Figure 7. Attenuation of cytokine-induced enhancement of TLR4 expression by butyrate-induced (A) and spontaneous differentiation (B). (A) HT-29 cells were differentiated by treatment with 3 mM butyrate for 48 h. Control and differentiated HT-29 cells were stimulated by 20 ng/ml TNF-α or 40 ng/ml INF-ɣ for 12 h. RNA were extracted and RT-PCR was performed with specific primers of indicated receptor components for TLR4 and β - actin. (B) Same experiment was done in HT-29 cells differentiated by culture 7 days after confluence.

7. The expressions of TLR4 are more pronounced in the lower portion of the crypts

We also wanted to know whether TLR4 was constitutively expressed in the human gut mucosa and whether this expression varies with differentiation status of epithelial cells. Immunohistochemical staining of human small intestine showed the expression of TLR4 was predominant in the lower portion of the crypts of Liberkühn (Fig. 8). While we observed that villous and colonic epithelium was largely devoid of TLR4 staining. These gradient TLR4 expressions through the crypt-villus axis *in vivo* confirm down-regulation of TLR4 expression along differentiation.

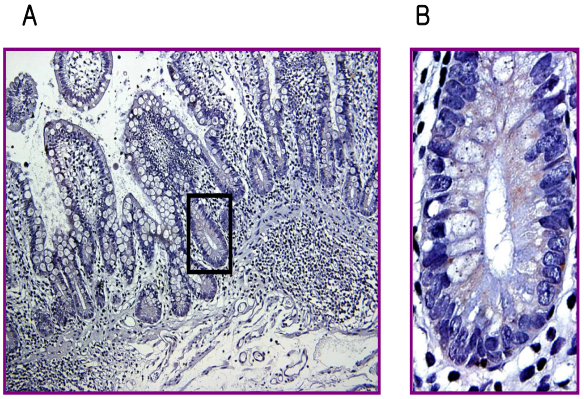

Figure 9. Spatial expression of TLR4 in human small intestine (A) Immunohistochemistry was performed on tissue of human small intestine using anti-TLR4 antibody as described in Materials and Methods (magnification, x100). (B)(x400)

IV. Discussion

Although some, but not all, intestinal epithelial cell lines are known to respond to LPS,[38] little is known about the relationship between LPS responsiveness and the expression of LPS receptors and other factors regulating LPS responsiveness. We demonstrated that HT-29 cells but not Caco-2 cells were responsive to LPS stimulation. We assume this difference in response to LPS stimulation between HT-29 and Caco-2 cells is resulted from the existence of TLR4.

The spontaneous differentiation of Caco-2 cells has been described extensively.[39-41] Similarly, the effects of butyrate on inducing markers of cell differentiation (AP activity) have been described in colon cancer cell lines.[33] In the present study, however, we described that HT-29 cells could also undergo spontaneous differentiation by post-confluence culture.

Several groups demonstrated that TLR4 is the signaling subunit of the LPS receptor using both genetic and biochemical approaches.[27, 42, 43] The luminal surface of the intestinal epithelium is exposed to many bacteria and their products continuously. And intestinal epithelial cells express LPS receptor, TLR4. Normally, however, commensal bacteria and their products including LPS do not elicit inflammatory response. Several investigators have explained the mechanism of lack of LPS response in intestinal cells. Cario et al. reported that the absence of membranous CD 14 expression may make intestinal epithelial cells hypo-responsive.[28] By several transfection experiments, Abreu et al. demonstrated that lack of expression of MD-2, a TLR4 co-receptor, could be an other explanation.[29] In this study, by *in vitro* and *in vivo* evidences, we report that down-regulation of TLR4 in differentiated epithelial cells might

be an another reason. A recent report have also demonstrated differentiation induced by treatment with butyrate or methotraxate (MTX) in HT-29 cells down-regulates TLR4 expression.[44]

In physiologic state, undifferentiated epithelial cells in crypt are usually isolated from luminal environment, because large amount of mucous substance and fluid are released from crypt,[45] and this hydrostatic pressure which pushes fluid toward the crypt orifice is strong enough to push out bacteria and their products. Impairment of epithelial integrity may cause crypt to be exposed to luminal content. It is conceptually possible that bringing TLR4 expressing cells of the crypt into contact with luminal bacteria and their products may perpetuate inflammatory responses.

Inflammatory bowel disease is characterized by uncontrolled inflammation in the absence of a recognized pathogen. Dysregulated production of Th1 cytokines, such as TNF-α and INF-γ , in the presence of commensal bacteria has been implicated in the pathogenesis of inflammatory bowel disease.[32, 46, 47] In this study, we found that TNF-α and INF-γ could enhance the sensitivity of LPS response assessed by production of IL-8 and up-regulate TLR4 expression in HT-29 cells. Our results showed a potential path of cooperatives between the innate and adaptive immune systems in intestinal epithelial cells. Cooperation between the innate and adaptive immune systems was also seen in the clearance of *Mycobacterium tuberculosis* from macrophage, which requires IFN-γ and TNF-α production by Th1 cells as well as LPS-dependent activation of TLR4 and TLR2.[48-51] As shown in results, TNF-α and INF-γ augmented TLR4 mRNA expression in HT-29 cells. Recently, similar observations were reported.[32] We also

observed not only TNF-α and INF-γ -induced augmentation of LPS response but also up-regulation of TLR4 expression in HT-29 cells were strongly attenuated by differentiation suggesting that differentiation causes down-regulation of TLR4 expression and LPS response in normal steady state as well as in inflamed state. The sustained nature of immune response in inflammatory bowel disease may have diverse cause. Poor barrier function and initial damage to surface epithelium may permit continued exposure of TLR4 expressing crypt cells to bacteria and their products from the lumen and pro-inflammatory cytokine-induced up-regulation of TLR4 may enhance LPS response.

In summary, we have identified that differentiation attenuates LPS induced IL-8 secretion in HT-29 cells and this attenuation is related with down-regulation of TLR4 expression. Our demonstration that cellular differentiation modulates intestinal epithelial cell responsiveness to LPS has an important biologic and clinical implication. It is likely that differences in immune responsiveness of intestinal epithelial cell to LPS exist along the villus-crypt axis. The relative abundance of undifferentiated cell in the inflamed mucosa may contribute to perpetuation of the inflammatory responses to LPS.

V. Conclusion

Intestinal epithelial cells control the host's local and systemic exposure to the toxic luminal environment by providing a physical barrier to uptake of bacterial antigens as well as by serving immunoregulatory function. Dysregulated immune response to commensal bacteria leads to chronic inflammation of intestine. The hypothesis that main mechanisms of immune regulation are compartmental location of epithelial cell and reduction of immune response according to degree of differentiation comes to the front. Although TLR4 has been identified as important components of innate immunity to LPS in intestine, little is known about regulation of TLR4.

We investigated the effects of differentiation on LPS induced IL-8 secretion and expression of TLR4, and concluded that down-regulation of TLR4 expression along with differentiation may be a mechanism by which intestinal epithelial cells protect against dysregulated immune and inflammatory response to commensal bacteria and their products. The physiologic impact of intestinal epithelial cell differentiation may include regulation of sensing luminal contents.

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국문요약

HT-29 세포의 분화에 의한 LPS에 대한 반응과 TLR4 발현의 감쇄

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소화관 상피세포가 단지 물리적인 장벽일 뿐만 아니라 면역 반응의 시작과 유지에 능동적인 역할을 하는 것으로 알려짐으로써 그 조절 기전에 대해 관심이 모아지고 있다. 정상적으로 미분화된 crypt의 장상피는 villus로 이행하면서 분화를 하게 되며, 이 과정에서 세포의 모양과 기능이 변하게 된다. 이러한 분화 과정 중에서 면역반응의 변화로서 IL-1β 에 대한 반응의 감소가 보고가 되었다. 장내 박테리아의 LPS는 염증성 장질환의 병인에서 중요한 역할을 할 것으로 생각이 되어 왔다. 최근에 들어서 LPS에 대한 염증반응을 매개하는 것으로 알려진 TLR4 수용체가 장상피세포에 존재하는 사실이 알려지면서 LPS에 대한 반응의 조절기전에 대해 관심이 모아지고 있다. 본 연구는 장상피세포의 분화가 LPS에 대한 IL-8 생성과 TLR4의 발현에 미치는 영향을 알아보고자 하였다. HT-29 세포를 분화시키는 방법으로는 butyrate를 처리하거나 confluence이후로 배지를 교체하며 지속적으로 배양하는 자발적 분화 방법을 이용하였고 분화의 정도를 확인하기 위해 AP 활성도를 측정하였다. LPS에 대한 IL-8 반응은 ELISA로 측정하였고, TLR 4 발현 변화를 보기 위해 western blot과 RT-PCR을 이용하였다. 정상 소장 조직에서 TLR4의 발현을 보기 위해 면역조직화학염색을 시행하였다.

1. HT-29세포는 LPS로 자극하였을 때 대해 용량의존적으로 반응하여 IL-8을 생산하였고, 100 ng/ml 농도에서 plateau를 이루었다.
2. Confluence 이후로부터 배양하는 기간(0-7일)이 길수록, 그리고

처리한 butyrate (0-3 mM)의 농도가 높을수록 AP 활성도가 증가하였다.

3. HT-29세포에서 분화를 유도하면 LPS에 대한 반응이 감소하였다.
4. HT-29세포에서 분화를 유도하면 TLR4 m-RNA와 단백질의 발현이 감소하였다.
5. TNF-α (20 ng/ml, 12시간)와 INF-ɣ (40 ng/ml, 12시간)의 전처치는 HT-29세포에서 LPS에 대한 반응을 증폭시키고 TLR4 m-RNA 발현을 증강시켰다.
6. TNF-α 와 INF-ɣ 에 의한 LPS에 대한 반응 증폭과 TLR4 발현 증강은 분화에 의하여 감쇄되었다.
7. 정상 소장조직에서 TLR4는 crypt의 하부에만 발현되었다.

이상의 결과로 HT-29세포에서 세포의 분화에 따른 LPS 반응의 감소는 LPS 수용체인 TLR4의 발현 감쇄와 연관된다고 생각하였다.

핵심되는 말: 분화, IL-8, LPS, TLR4, butyrate