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Epigenetic Regulation of TLR4 Gene Expression in Intestinal Epithelial Cells for the Maintenance of Intestinal Homeostasis

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Intestinal epithelial cells (IECs) are continuously exposed to large numbers of commensal bacteria but are relatively insensitive to them, thereby averting an excessive inflammatory reaction. In this study, we show that the low responsiveness of human IEC lines to LPS was mainly brought about by a down-regulation of TLR4 gene transcription. Additionally, the presence of an IEC-specific repressor element in the 5′ region of the TLR4 gene and binding of a NF to the element was shown. The transcription factor ZNF160, which was expressed more abundantly in a LPS-low responder IEC line than in a LPS-high responder IEC line, repressed TLR4 gene transcription. ZNF160 is known to interact with the scaffold protein KAP1 via its N terminus to recruit histone deacetylase. Histone deacetylation, as well as DNA methylation, at the 5′ region of the TLR4 gene was significantly higher in LPS-low responder IEC lines than in a monocyte line or a LPS-high responder IEC line. It was demonstrated that TLR4 gene transcription was repressed by these epigenetic regulations, which were, at least in part, dependent on ZNF160. Down-regulation of TLR4 gene expression by these mechanisms in IECs possibly contributes to the maintenance of homeostasis in the intestinal commensal system. The Journal of Immunology, 2009, 183: 6522–6529.

The intestinal immune system, which is the largest immune system in the body, plays an essential role in the maintenance of health. In addition to food, pathogenic bacteria and viruses enter the intestinal tract through the oral cavity. On the other hand, an enormous number of commensal bacteria inhabit the intestinal tract. The intestinal immune system accurately recognizes these different organisms, discriminates between safe/beneficial and dangerous components, and attacks only those that are hazardous to the host.

Lately, the crucial role of the symbiosis between the commensal bacteria and the intestinal immune system in maintaining good health has attracted considerable attention. For instance, the development of GALTs such as Peyer’s patches and the induction of oral tolerance are known to be impaired or delayed in germ-free animals (1–5). Moreover, a correlation between the incidence of atopic eczema in children and the composition of their intestinal microbiota has been reported (6–11). The following phenomena observed specifically in the intestinal mucosa are thought to be involved in establishment and maintenance of the symbiosis, although the precise underlying mechanisms have not yet been fully elucidated. First, maturation of a specific population of dendritic cells expressing CD103, which preferentially induce T cells with regulatory properties, is accelerated in the intestine (12, 13). Second, the commensal bacteria regulate the differentiation of effector T cells and achieve appropriate Th1/Th2 and Th17/regulatory T cell balances (14, 15). Third, production of IgA against the commensals is involved in keeping the number of the commensals at an appropriate level (16). Additionally, it is also important that intestinal epithelial cells (IECs),3 which form the boundary between the lumen and the internal milieu, acquire hyporesponsiveness to the commensals, because the luminal surface of the epithelium is continually exposed to the commensals or their components.

IECs not only physically separate luminal contents from the internal milieu but also actively participate in the immune reactions as a frontline defense in the mucosal immune system. IECs have been shown to receive stimulation from intestinal commensal bacteria through TLRs, the pattern recognition receptors for microbial components, to maintain their homeostasis (17). However, to prevent triggering excessive inflammatory reactions, they do not respond in a sensitive manner to the commensals. Specifically, IECs contribute to the maintenance of intestinal homeostasis by partially tolerating the commensals and regulating mucosal inflammation. Decreased expression of specific TLRs and accessory molecules such as MD2 in IECs has been reported as one of the mechanisms controlling the hyporesponsiveness (18, 19). Additionally, the expression of Toll-interacting protein, a negative regulator of intracellular signals from TLRs, is known to be increased in IECs (18). In contrast, excessive responses to the commensals and increased expression of specific TLRs, including TLR4, are often observed in patients with inflammatory bowel disease (20). Therefore, expression of these molecules is thought to be regulated by cell type-specific mechanisms in IECs to maintain the intestinal symbiosis. Here we have analyzed the regulatory mechanisms of TLR4 gene expression in IECs to elucidate one of the mechanisms for maintaining the intestinal homeostasis.

3 Abbreviations used in this paper: IEC, intestinal epithelial cell; DN, dominant negative; qPCR, quantitative PCR; ChIP, chromatin immunoprecipitation; 5-aza-dC, 5-aza-2′-deoxycytid ine; TSA, trichostatin A; siRNA, small interfering RNA; KRAB, Kruppel-associated box; KAP, KRAB-associated protein; IC50, interferon consensus sequence-binding protein.

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**Materials and Methods**

**Cell culture**

The human epithelial colon adenocarcinoma cell lines Caco-2 and HT-29 and the human epithelial colon carcinoma cell lines HCT 116 and T84, as well as the human monocyte line THP-1, were purchased from DS Pharma Biomedical. The human epithelial colon adenocarcinoma cell line SW480 was provided by the Cell Resource Center for Biomedical Research and Development, Aichi Cancer Research Center, Nagoya University (Miyaichi, Japan). Caco-2 cells were cultured in Eagle’s MEM (Nissui) supplemented with nonessential amino acids (Invitrogen). HCT 116 and HT-29 cells were cultured in McCoy’s 5a medium (MP Biomedicals). T84, SW480, and THP-1 cells were cultured in a 1/1 mixture of Ham’s F12 (Nissui) and DMEM (Nissui); a 1/1 mixture of L15 (MP Biomedicals) and DMEM, and RPMI 1640 (Nissui), respectively. All media were supplemented with 10% (v/v) FBS (Biowest), 100 U/ml of penicillin, and 100 µg/ml of streptomycin (Nissui), respectively.

**Measurement of IL-8 production**

Cells were stimulated for 18 h with 0.1–1000 ng/ml ultra-pure Escherichia coli K12 LPS (InvivoGen), which is guaranteed to only activate the TLR4 pathway. Concentrations of secreted IL-8 in the culture supernatants were measured with a human IL-8 ELISA kit (BioSource International) or Quanti-tikin human CXCL/L-8 (R&D Systems) according to the manufacturers’ instructions.

**Measurement of NF-κB activation by a reporter gene assay**

Cells were transfected with 1.5 µg of NF-κB reporter plasmid carrying NF-κB binding sites upstream of the luciferase gene using FuGene HD transfection reagent (Roche). Alternatively, cells were cotransfected with 1.5 µg of NF-κB reporter plasmid and 1.5 µg of an expression plasmid encoding the dominant-negative (DN) form of MyD88. The plasmid phRL-CMV (1.25 ng/well; Promega) carrying the Renilla luciferase gene under the control of the CMV promoter was introduced to normalize the transfection and cell lysis efficiencies in every experiment. Four hours after transfection, ultra-pure E. coli K12 LPS (InvivoGen) was added to the cells. After culturing the cells for an additional 20 h, the cells were harvested. Cell lysis and determination of luciferase activity were conducted using a dual-luciferase assay kit (Promega) according to the manufacturer’s instructions. Luminescence was measured with a TriStar LB941 luminometer (Berthold).

**Quantitative RT-PCR (qRT-PCR)**

Total RNA was prepared from each cell line using an RNAeasy Mini Kit (Qiagen) or a High Pure RNA isolation kit (Roche), and the first-strand cDNA synthesis was then conducted using SuperScript II reverse transcriptase (Invitrogen). Messenger RNA expression was quantified by real-time PCR with a LightCycler 480 (Roche) using SYBR Green I Master reagent (Roche). The sequences of the synthetic oligonucleotides used as primers for PCR with a LightCycler 480 (Roche) using SYBR Green I Master reagent (Roche) were as follows: for detection of TLR4 gene expression, forward: 5′-TTCAGTATATTCCATTCTGAAA-gcggtac-3′ and reverse: 5′-CTGACCCAGGTCCTCTTCACACCTGCAC-3′; for detection of p155 gene expression, forward: 5′-GGATGAGTGGAACTTGGAGCCTAAGGGTGTTGTTGTTGATCTGCACTGGAGGAAAGGTGCCTTCTACAC-3′ and reverse: 5′-TTCACACACCTCTTGCTGTGA-3′.

**Measurement of transcriptional promoter activity by a reporter gene assay**

The reporter plasmid pGL-TLR4−1013/+118 (0.7 µg/ml) was introduced into cells using FuGene HD transfection reagent (Roche). For overexpression experiments, cells were cotransfected with 0.5 µg of the reporter plasmid pGL-TLR4−1013/+118 and 0.5 µg of pcDNA3.1-ZNF160 sense, or pcDNA3.1-nt sense plasmid. The plasmid phRL-CMV (1.25 ng/ml) was introduced to normalize the transfection and cell lysis efficiencies in every experiment. After 20–24 h of culture, cells were harvested and washed with PBS. Cell lysis and determination of luciferase activity were performed as described above.

**FACS**

After treatment with Fc block (anti-mouse CD16/CD32; BD Biosciences), cells were incubated with anti-human TLR4 mAb (clone HTA125; eBioscience) or anti-TLR2 mAb (clone TL2.1; eBioscience) using FACSCanto (BD Biosciences).

**Immunoprecipitation**

Cells were washed with ice-cold PBS and incubated on ice for 30 min in lysis buffer (20 mM Tris (pH 7.6), 1% Nonidet P-40, 60 mM octyl-β-glucoside, 2 mM PMFS, 10 µg/ml aprotinin, 2 mM ml leupeptin, 2 µg/ml pepstatin). Cell lysates were immunoprecipitated with anti-TLR4 mAb (clone HTA125; eBioscience) or anti-TLR2 mAb (clone TL2.1; eBioscience) using protein A-Sepharose (GE Healthcare). Immunoprecipitated proteins were detected by immunoblotting with anti-TLR4 or anti-TLR2 mAb. Part of each immunoprecipitated cell lysate was subjected to immunoblotting with...
FIGURE 1. Responsiveness of human IEC lines to LPS correlates with their TLR4 mRNA expression levels. A, Several human IEC lines (Caco-2, HCT 116, T84, HT-29, and SW480) and a human monocyte line (THP-1) were stimulated with 0.1–1000 ng/ml LPS for 18 h. Secretion of IL-8 into the culture supernatant was measured by ELISA. IL-8 production is represented on the graph by subtracting the concentrations in the supernatant of unstimulated cells. Results are represented as means ± SD of two independent experiments. N.D. indicates not detected. B, Activation of NF-κB by stimulation with LPS was measured by reporter assays. Each cell line was cotransfected with a NF-κB reporter plasmid and an expression plasmid carrying MyD88 DN or an empty vector control and stimulated with the indicated concentrations of LPS. The fold increase in luciferase activity relative to that of unstimulated cells transfected with the empty vector control is shown. Results are represented as means ± SD of three independent experiments. C, TLR4 mRNA expression in each cell line was determined by qRT-PCR. Relative values normalized using GAPDH mRNA levels are given. Results are represented as means ± SD of three independent experiments. D, A TLR4 siRNA expression plasmid or a control siRNA expression plasmid was introduced into SW480 cells. After selection of the transfected cells with puromycin, TLR4 mRNA expression was determined by qRT-PCR. Relative values normalized using GAPDH mRNA levels are given. Results are expressed as means ± SD of four independent experiments: —, no transfection; TLR4-transfected with the TLR4 siRNA expression plasmid; control, transfected with the control siRNA expression plasmid; *, p < 0.001; E, SW480 cells were transfected with the TLR4 siRNA expression plasmid (filled bars) or the control siRNA expression plasmid (open bars). After selection with puromycin, the transfected cells were stimulated with 0.1–1000 ng/ml LPS for 18 h to measure IL-8 secretion into the culture supernatant by ELISA. IL-8 production was calculated by subtracting the concentrations in the supernatant of unstimulated cells from those of stimulated cells. Results are expressed as means ± SD of four independent experiments. *, p < 0.05 and **, p < 0.005 significantly different from control stimulated with the same concentration of LPS.

anti-β-actin mAb (Abcam) to quantify the amounts of proteins present in each lysate before immunoprecipitation.

Nuclear extract preparation

Nuclear extract was prepared from Caco-2 and THP-1 cells as described previously (21).

EMSA

Double-stranded DNAs were prepared as probe A, B, and C by annealing Alexa 647-labeled synthetic oligonucleotides. Nucleotide sequences of the probes were as follows: probe A, 5′-GACTGTATATGGAGAGAGCC-3′; probe B, 5′-CCCTGAAAGAGGTATGTAAGTAGA-3′; probe C, 5′-GTAGAATGAGGTCATTATGGTG-3′.

Sixteen micrograms of nuclear extract and 5 pmol of DNA probe were incubated at room temperature in 10 mM HEPES buffer (pH 7.9) containing 400 ng of poly(dI:dC), 1 mM MgCl2, 30 mM KCl, 1 mM DTT, and 5% glycerol for 20 min. The mixtures were subjected to electrophoresis on 4% polyacrylamide gels at 120 V for 2.0–2.5 h in 0.25 TBE buffer (22.5 mM Tris, 22.5 mM boric acid, 0.5 mM DTT). Fluorescence was detected using a Typhoon 9410 (GE Healthcare).

cDNA subtraction

cDNAs of the genes that were expressed more abundantly in Caco-2 cells than in SW480 cells were enriched with a PCR-Select cDNA subtraction kit (Clontech) according to the manufacturer’s instructions. Obtained cDNA candidates were inserted into the pcDNA3.1/V5-His-TOPO vector (Invitrogen) to yield their expression plasmids. The expression plasmids were introduced into SW480 cells as groups of five clones together with the reporter plasmid pGL3-TLR4-1013/H11032 to yield their expression plasmids. The expression plasmids carrying 400 ng of poly(dI:dC), 1 mM MgCl2, 30 mM KCl, 1 mM DTT, and 5% glycerol for 20 min. The mixtures were separated by electrophoresis on 4% polyacrylamide gels at 120 V for 2.0–2.5 h in 0.25 TBE buffer (22.5 mM Tris, 22.5 mM boric acid, 0.5 mM DTT). Fluorescence was detected using a Typhoon 9410 (GE Healthcare).

Bisulfite conversion reaction

Genomic DNA was prepared from cells using a PureLink Genomic DNA Mini kit (Invitrogen) according to the manufacturer’s instructions. To analyze methylation of CpG motifs, 400 ng of genomic DNA was denatured and subjected to ChIP assays employing anti-acetyl histone H3 Ab. The 5′-ACTATCG AAGTCTTTACCCCTTAC-3′ and 5′-CAGGGACTTCAACTGTGA-3′ as primers. The GAPDH promoter region was amplified as a control using primers with the sequences 5′-TACTAGCGTTTACCGCG-3′ and 5′-TGAGAGAGAGACGAGAGAGGA-3′.

For the ZNF160 overexpression experiment, cells were transfected with pcDNA3.1-ZNF160 sense or pcDNA3.1-empty using FuGene HD transfection reagent. After 8 h, G418 (Sigma-Aldrich) was added to select for transfected cells. Cells were cultured for an additional 40 h, harvested, and subjected to ChIP assays employing anti-acetyl histone H3 Ab. The 5′ region of the TLR4 gene and that of the GAPDH gene in the immunoprecipitated DNA were quantified by real-time PCR analyses using the same primers described above.

Bisulfite conversion reaction

Genomic DNA was prepared from cells using a PureLink Genomic DNA Mini kit (Invitrogen) according to the manufacturer’s instructions. To analyze methylation of CpG motifs, 400 ng of genomic DNA was denatured...
at 98°C for 10 min. modified by conversion reagent at 64°C for 2.5 h, and then purified using a MethylCode bisulfite conversion kit (Invitrogen). The 5’ region of the TLR4 gene was amplified by PCR from the modified genomic DNA. The sequences of the synthetic oligonucleotides used as PCR primers are as follows: forward, 5’-GGTAGAGGTAGATGATTACGGGG-3’; reverse, 5’-CCCCAATAACTACCTCTAATACCCCT-3’.

Following purification of PCR products, they were cloned into the pCR2.1 vector for sequencing. Nucleotide sequences of 14–16 clones for each cell line were analyzed.

Inhibition of histone deacetylase and DNA methyltransferase

Caco-2 cells were treated with 10 μM 5-aza-2’-deoxycytidine (5-Aza-dC; Calbiochem) for 4 days, with 80 nM trichostatin A (TSA; Cayman Chemical) for 24 h, or with TSA and 5-Aza-dC for 24 h after treatment with 5-aza-dC alone for 3 days. Cells were then harvested to obtain total RNA. TLR4 mRNA expression was quantified by qRT-PCR as described above.

Results

Responsiveness of human IEC lines to LPS is down-regulated mainly by repressing TLR4 gene transcription

Various human IEC lines were first analyzed for their ability to respond to stimulation through TLR4. Five human IEC lines (Caco-2, HT-116, TH-29, SW-480, and a control monocyte line (THP-1) were stimulated with LPS to measure IL-8 secretion into the culture supernatant (Fig. 1A). Most IEC lines, with the exception of SW-480, showed low responsiveness to LPS compared with the monocyte line THP-1. Caco-2 and HT-116 cells barely responded, and HT-29 and T84 cells weakly responded to LPS stimulation. In contrast, product of IL-8 from SW-480 cells was similar to, or even higher than, IL-8 production from the monocyte line THP-1. The MyD88 dependency of these responses was further examined by NF-κB reporter assays (Fig. 1B). Consistent with the results for IL-8 production, activation of NF-κB was observed in SW-480 and THP-1 cells when stimulated with LPS, while it was hardly detected in Caco-2 cells. Activation of NF-κB was inhibited by exogenous expression of the DN form of MyD88 in SW-480 and THP-1 cells, indicating that the responses to LPS were dependent on MyD88. The TLR4 mRNA expression in each cell line was determined by qRT-PCR (Fig. 1C). The amounts of TLR4 mRNA almost exactly correlated with the LPS responsiveness of each cell line. Furthermore, TLR4 mRNA expression was reduced to about one-third in SW-480 cells transfected with a TLR4 siRNA expression plasmid when compared with the cells transfected with a control siRNA expression plasmid (Fig. 1D), which led to a significant decrease in IL-8 production upon stimulation with LPS (Fig. 1E). These results show that LPS sensitivity is regulated by TLR4 gene expression in IECs. Next, the cell surface expression of TLR4 and the transcriptional enhancing activity of the 5’ region of the gene upstream of the translation start codon were analyzed in each cell line (Fig. 2, A and B). The cell surface expression of TLR4 was undetectable on Caco-2 and HCT 116 cells. On the other hand, HT-29 and T84 cells expressed a small amount of cell surface TLR4. SW-480 cells expressed a higher level of cell surface TLR4 than did HT-29 and T84 cells. The highest cell surface expression of TLR4 was observed on the monocyte line THP-1. The transcriptional promoter activity of the 5’ region of the TLR4 gene was, in order, THP-1, SW-480 > HT-29, T84 > Caco-2, HCT 116. Intracellular protein expression of TLR4 in each cell line was further analyzed by immunoprecipitation (Fig. 2C). TLR4 protein was barely detected in Caco-2 cells but was detected at higher levels in SW-480 and THP-1 cells, while TLR2 was apparently detected in all cell lines. These results indicate that expression of the TLR4 gene is mainly regulated at the transcriptional level, although additional posttranscriptional regulation also seems to be present. Collectively, it was shown that the responsiveness of IECs to LPS is down-regulated mainly by suppressing transcription of the TLR4 gene.

Nucleotide −489/−428 region of the TLR4 gene contains an IEC-specific repressor element and is recognized by an IEC-specific NF

To further examine the mechanisms of transcriptional repression of the TLR4 gene in IECs, cis-acting elements in the 5’ region of the TLR4 gene were compared between Caco-2 and THP-1 cells. For this purpose, a series of deletion constructs were employed for luciferase assays. Deletion of the nt −675/−428 region increased the luciferase activity in Caco-2 cells, while it hardly affected the activity in THP-1 cells, suggesting that this region contains an IEC-specific repressor element (Fig. 3A). Moreover, this region was suggested to contain a responsive element that is repressed by some microbe, because the transcriptional enhancing activity of nt −675/−188 but not of nt −427/−188 was decreased by the synthetic TLR2 ligand, Pam3CSK4 (Fig. 3B). Further mapping of the repressor element in the nt −675/−428 region by luciferase assays using deletion constructs in Caco-2 cells revealed that the nt −489/−428 region acts as a repressor element (Fig. 3C). We next examined the presence of a NF binding to this region by EMSA using three oligonucleotide probes covering this region and nuclear extracts prepared from Caco-2 and THP-1 cells. The shifted band indicated by an arrow in Fig. 3D appeared when the nuclear extract
FIGURE 3. Nucleotide –489/—428 region contains an IEC-specific repressor element and is recognized by an IEC-specific NF. A, cis-Acting elements in the 5′ region of the TLR4 gene were determined by a reporter gene assay using a series of deletion constructs in Caco-2 and THP-1 cells. Luciferase activities relative to that of pGL3-TLR4–1013/+188 are shown. Results are expressed as means ± SD of three independent experiments. PU.1 and ICSBP binding sites reported by Rehli et al. (31) and enhancer and repressor elements suggested from our study are schematically drawn. B, Caco-2 cells were transfected with the reporter plasmid pGL3-TLR4–1013/+188, pGL3-TLR4–675/+188, pGL3-TLR4–427/+188, or pGL3-TLR4–218/+188 for a transient expression assay in the presence or absence of 1 μg/ml Pam3CSK4. Luciferase activities relative to those without Pam3CSK4 treatment are shown in each graph. Results are expressed as means ± SD of two independent experiments. C. For further mapping of a repressor element in the nt –675/—428 region, Caco-2 cells were transfected with a series of deletion constructs for a transient expression assay. Luciferase activities relative to those of pGL3-TLR4–1013/+188 are shown. Results are expressed as means ± SD of three independent experiments. D, EMSA was performed using three double-stranded oligonucleotide probes corresponding to the regions nt –492/—468, nt –472/—448, and nt –452/—428 of the TLR4 gene and nuclear extracts prepared from Caco-2 and THP-1 cells. Results are representative of three independent experiments.

from Caco-2 cells but not from THP-1 cells was added, indicating that a NF that was specifically expressed in Caco-2 cells bound to the nt –452/—428 region. Collectively, these results suggest that a specific NF binds to the nt –489/—428 region and represses TLR4 gene transcription in IECs.

**ZNF160 represses TLR4 gene transcription in IECs**

To elucidate the molecules involved in the transcriptional repression of the TLR4 gene in IECs, we screened a human cDNA library, using the PCR-select subtraction method, for molecules that were expressed more abundantly in the LPS-low responder IEC line Caco-2 than in the LPS-high-responder IEC line SW480. The obtained cDNA clones were screened again to identify molecules that repressed transcription of the TLR4 gene when overexpressed in SW480 cells by a transient expression assay. Out of the 360 cDNA clones selected by subtraction, 7 clones, including 6 clones that encoded 3 different molecules and 1 clone that did not encode the protein in frame, decreased the TLR4 promoter activity to less than half. One of the three identified molecules that were encoded by cDNAs in frame and repressed activation of the TLR4 gene promoter was ZNF160. As shown in Fig. 4A, it was confirmed that ZNF160 mRNA expression was higher in Caco-2 cells than in SW480 cells by qRT-PCR analyses. ZNF160 is a Kruppel-related zinc finger protein characterized by the presence of the Kruppel-associated box (KRAB), a potent repressor of transcription, at the N terminus (23). Overexpression of ZNF160 decreased the transcriptional promoter activity of the TLR4 gene in SW480 cells (Fig. 4B), whereas, interestingly, it up-regulated the promoter activity in the human monocyte line THP-1 (Fig. 4C). Furthermore, ZNF160 repressed the transcriptional enhancing activity of nt –675/+188 but not of nt –427/+188 in SW480 cells, indicating that ZNF160 acts through the nt –675/—428 region (Fig. 4, D and E).

**TLR4 gene transcription is down-regulated by epigenetic modification including histone deacetylation and DNA methylation in IECs**

KRAB domains have been reported to recruit KRAB-associated protein (KAP) 1 when tethered to DNA via their zinc finger motifs (24, 25). KAP1 directly binds to KRAB and functions as a scaffold for the formation of a multimolecular complex comprising histone deacetylases, which induces transcriptional repression through the formation of heterochromatin (26–28). In addition to histone deacetylation, it was recently reported that the KRAB domain can trigger de novo DNA methylation (29). Histone acetylation and DNA methylation are known to mediate epigenetic regulation of gene expression. To investigate if TLR4 gene expression is controlled by these epigenetic mechanisms in IECs, acetylation of histones interacting with the 5′ region of the TLR4 gene was analyzed by ChIP assays using anti-acetyl histone H3 Ab. As shown in Fig. 5, acetylated histones interacting with the 5′ region of the TLR4 gene were significantly lower in Caco-2 and HCT 116 cells, both of which barely express TLR4, than in SW480 and THP-1 cells, indicating that the histones interacting with the 5′ region of the TLR4 gene were highly deacetylated in LPS-low responder IEC lines. DNA methylation of 11 CpG motifs existing in the 5′ region of the TLR4 gene (from nt –69 to nt +277) was next analyzed by the bisulfite conversion reaction. As is shown in Fig. 6, the number of methylated CpG motifs was significantly higher in Caco-2 and HCT 116 cells than in SW480 and THP-1 cells.
that DNA methylation and histone deacetylation are cooperatively involved in the repression of TLR4 gene transcription (Fig. 7A). Collectively, these results show that TLR4 gene transcription is down-regulated by epigenetic modification including histone deacetylation and DNA methylation in LPS-low responder IEC lines.

The epigenetic modification is, at least in part, dependent on ZNF160

To analyze the involvement of ZNF160 in epigenetic regulation, ChIP assays of the endogenous TLR4 gene were performed employing SW480 cells transfected with a ZNF160 expression plasmid or an empty vector control. Acetylation of histones that interacted with the 5' region of the TLR4 gene was significantly reduced by the overexpression of ZNF160, while acetylation of histones that interacted with the 5' region of the GAPDH gene was not affected (Fig. 7, B and C). The results indicate that epigenetic

![FIGURE 4](https://example.com/fig4.jpg)

FIGURE 4. ZNF160, one of the molecules expressed more abundantly in the LPS-low responder IEC line Caco-2 than in the LPS-high responder IEC line SW480, represses transcription of the TLR4 gene. A, ZNF160 mRNA expression in Caco-2 and SW480 cells was determined by qRT-PCR. Relative values normalized using GAPDH mRNA levels are given. Results are expressed as means ± SD of three independent experiments. *, p < 0.05; **, p < 0.005.

![FIGURE 5](https://example.com/fig5.jpg)

FIGURE 5. Histones interacting with the 5' region of the TLR4 gene are deacetylated in LPS-low responder IEC lines. ChIP assays of endogenous TLR4 genes in IEC lines were performed employing anti-acetyl histone H3 Ab. Rabbit IgG was used as a control. The 5' regions of the TLR4 gene, and of the GAPDH gene as a control, were amplified by PCR from the immunoprecipitated chromatin. The "input" lanes represent the results of PCR using diluted fractions of nonimmunoprecipitated chromatin as templates. Results are representative of three independent experiments.

![FIGURE 6](https://example.com/fig6.jpg)

FIGURE 6. The 5' region of the TLR4 gene is highly methylated in LPS-low responder IEC lines. DNA methylation of 11 CpG motifs existing in the 5' region of the TLR4 gene (nt –69/+277) was analyzed by the bisulfite conversion reaction. The modified DNA was amplified by PCR, cloned, and sequenced for 14–16 independent clones. A, Filled squares indicate methylated CpG motifs, and open squares indicate unmethylated CpG motifs. B, Mean numbers of methylated CpG motifs in the nt –69/+277 region and SD in each cell line are shown. *, p < 1 × 10^{-7} and **, p < 5 × 10^{-9}.

![FIGURE 7](https://example.com/fig7.jpg)

FIGURE 7. Epigenetic modification is involved in repression of TLR4 gene transcription and is, at least in part, dependent on ZNF160. A, Caco-2 cells were treated with 10 μM 5-aza-dC (DNA methyltransferase inhibitor) for 4 days or with 80 nM TSA (histone deacetylase inhibitor) for 24 h. TLR4 mRNA expression was analyzed by qRT-PCR. Relative values, normalized using GAPDH mRNA levels, are expressed. Results are represented as means ± SD of three independent experiments. *, p < 0.05.
modulation at the 5’ region of the TLR4 gene is, at least in part, dependent on ZNF160.

Discussion

In this report, we show that TLR4 gene transcription is epigenetically suppressed in IECs to prevent excessive inflammatory responses. This means that epigenetic regulation of TLR4 gene expression in IECs can act as one mechanism for maintaining intestinal homeostasis by suppressing excessive responses to the commensals and regulating mucosal inflammation in the gut. Epigenetic information is encoded by differential methylation of DNA on cytosines and by proteins associating with DNA such as histones, which may be modified covalently by acetylation, methylation, phosphorylation, and/or ubiquitination. Since this epigenetic information is heritable beyond cell division, the importance of epigenetic regulation is established especially in the fields of developmental and cancer biology. This is the first report to describe the involvement of epigenetic regulation of transcription in the maintenance of the intestinal commensal system. Although the intestinal epithelium is known to be continuously renewed by rapid turnover of IECs, there are many reports supporting that the characteristics of IECs including their tolerated responses to the commensals are inherited by renewed cells, raising the possibility that epigenetic regulation as seen in our study is involved in such inheritance. These basic mechanisms may work commonly in other commensal systems in specific tissues in our body such as the skin epidermis or the mucosa of the oral cavity, in addition to the intestine, all of which are continuously exposed to nonpathogenic microbes. Zampetaki et al. recently reported that murine TLR4 gene expression is epigenetically repressed in embryonic stem cells but not in embryonic stem cell-derived differentiated smooth muscle cells (30). Epigenetic repression may be released in differentiated cells except specific types of cells including IECs.

Rehli et al. reported that transcription factors of PU.1 and IFN consensus sequence-binding protein (ICSBP) regulate human TLR4 gene expression in myeloid cells through elements just upstream of the transcription start site (31). As shown in Fig. 3A, additional enhancer elements seem to be present further upstream of the PU.1 and ICSBP binding sites identified by Rehli et al. Moreover, the presence of an IEC-specific repressor element in the 5’ region of TLR4 gene and an IEC-specific NF binding to the element has been suggested. We tried to identify the transcription factor forming the Caco-2-specific band shown in Fig. 3D but were unsuccessful. Since it was found that ZNF160 acts through the nt −675/−428 region (Fig. 4, D and E), it was thought that ZNF160 binds to this region. However, in vitro translation products of ZNF160 did not bind to the double-stranded DNA probe containing this region in EMSA (data not shown). Some modification of the ZNF-160 protein may be required for DNA binding, or, alternatively, ZNF-160 may indirectly bind to this region through another DNA binding factor. Stimulation with Pam3CSK4 repressed the transcriptional enhancing activity of the 5’ region of the TLR4 gene through the nt −675/−428 region (Fig. 3B). It is unclear at present whether specific microbial components modify transcription of the TLR4 gene, although LPS may not affect transcriptional activation because expression of TLR4 recognizing LPS was undetectable on Caco-2 cells (Fig. 2A).

The epigenetic regulation of TLR4 gene expression has been found to be partly dependent on ZNF160, a repressive transcription factor possessing the KRAB domain, which has been reported to recruit histone deacetylase through the scaffold protein KAP1. KAP1 is characterized by the presence of a RING finger, B boxes, a coiled-coil region, a PHD finger, and a bromodomain; the first three of these motifs are both necessary and sufficient for homologimerization and direct binding to KRAB (24, 25). A reduction in acetylation of histones that interact with the 5’ region of the TLR4 gene is thought to be dependent on histone deacetylase, which is recruited by ZNF160 through KAP1. Similarly, an increase in methylation of the 5’ region of the TLR4 gene in IECs is thought to be dependent on ZNF160. However, the contribution of ZNF160 to the regulation of TLR4 gene expression seems to be rather small compared with the large contribution of epigenetic modifications to the repression of TLR4 gene expression. Actually, overexpression of ZNF160 only suppressed transcriptional promoter activity of the TLR4 gene to about a half in SW480 cells (Fig. 4B), while inhibitors of histone deacetylase and DNA methyltransferase together increased TLR4 gene expression by ~10-fold in Caco-2 cells (Fig. 7A). Additionally, overexpression of ZNF160 in the monocyte line increased transcriptional promoter activity, while overexpression in the IEC line suppressed transcription (Fig. 4, B and C). These results suggest that ZNF160 and an additional cell type-specific regulatory factor cooperatively repress transcription of the TLR4 gene in IECs. Recently, a single nucleotide polymorphism with strong association to ileal Crohn’s disease was mapped to an intergenic region that is flanked on the centromeric side by a gene encoding another zinc finger protein, ZNF365 (32). Although the functions of ZNF365 are little known, specific members of the C2H2 zinc finger proteins, which constitute the largest class of transcription factors in humans, may play a role in the maintenance of intestinal homeostasis.

LPS responsiveness of IECs depends on TLR4 expression levels as shown in Fig. 1E. Additionally, expression of MD-2 is thought to be another important factor that determines the LPS responsiveness because optimal LPS recognition by TLR4 requires MD-2 as a coreceptor. It has been reported that MD-2 expression in healthy, normal intestinal mucosa is minimal, while it is increased in active inflammatory bowel disease colitis (33, 34). Moreover, inhibition of CpG methylation and histone deacetylation was shown to result in increased mRNA expression of MD-2 gene in IECs just recently (35). TLR4 and MD-2 expression may be down-regulated in part by common or related mechanisms in IECs. On the other hand, expression of TLR4 and TLR2 is thought to be regulated at different stages. TLR4 expression has been found to be regulated mainly at the transcriptional level because its cell surface expression correlates relatively well with mRNA expression levels in each cell line. However, regulation at the posttranslational level is also thought to be present, as a difference in the cell surface TLR4 expression was seen between SW480 and THP1 cells despite these cells having almost the same mRNA expression levels (Figs. 1 and 2). Since IL-8 production in response to LPS stimulation was rather higher in SW480 cells than in THP-1 cells, a certain extent of cell surface TLR4 expression seems to be sufficient to respond to LPS. On the other hand, expression of TLR2 seemed to be regulated mainly at the posttranslational level because its mRNA and intracellular protein expression was not apparently different between the IEC lines and the monocyte line, but its cell surface expression was much lower in the IEC lines than in the monocyte line (Fig. 2C and our unpublished data). Intracellular transport of TLR2 protein to the cell surface is thought to be inhibited by a specific mechanism in IECs. Further study should allow elucidation of the entire regulatory mechanism underlying transcription of symbiosis-associated genes, including TLR4 in IECs and its contribution to regulation of mucosal inflammation triggered by IECs, and, as a result, maintenance of the intestinal commensal system.

Disclosures

The authors have no financial conflicts of interest.
References


