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Lipopolysaccharide Activates Nuclear Factor-KappaB through Toll-Like Receptors and Related Molecules in Cultured Biliary Epithelial Cells

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SUMMARY: To clarify the innate immunity of the intrahepatic biliary tree, we examined expression of Toll-like receptors and intracellular signalings in biliary epithelial cells in response to bacterial components by using cultured biliary epithelial cells (murine biliary cells and human cholangiocarcinoma cell lines). The expression of Toll-like receptors in cultured cells was examined by reverse transcription and PCR and immunohistochemistry. Intracellular signalings after Toll-like receptors activation by lipopolysaccharide was examined by analysis of nuclear factor (NF)-kappaB activation and inhibition studies using inhibitors for NF-kappaB and mitogen-activated protein kinase and blocking antibody. The mRNAs of Toll-like receptors 2, 3, 4, and 5, and related molecules (MD-2, MyD88, and CD14) were detected, and their proteins were expressed in cultured cells. Lipopolysaccharide was shown to bind to the cell surface of cultured cells. Lipopolysaccharide treatment induced the production of TNF-alpha, and nuclear translocation of NF-kappaB and increased NF-kappaB-DNA binding in cultured cells. This induction of TNF-alpha was partially inhibited by anti–Toll-like receptor 4 antibody. The nuclear translocation and increased binding of NF-kappaB by lipopolysaccharide were blocked by addition of MG132, an inhibitor of NF-kappaB. In conclusion, lipopolysaccharide appears to form a receptor complex of CD14, Toll-like receptor 4, MD-2, and MyD88 in cultured biliary epithelial cells and seems to regulate activation of NF-kappaB and synthesis of TNF-alpha. The recognition of pathogen-associated molecular patterns using Toll-like receptors and related molecules in biliary epithelial cells, which is demonstrated in this in vitro study, may participate in an immunopathology of the intrahepatic biliary tree in vivo. (*Lab Invest 2003, 83:1657–1667*).

Bacterial components such as lipopolysaccharide (LPS) and lipoteichoic acids (LTAs), which are called pathogen-associated molecular patterns (PAMPs), are known to activate the innate immunity and also to induce inflammatory reactions (Anderson, 2000; Chuang and Ulevitch, 2001; Kaisho and Akira, 2002). The Toll-like receptor (TLR) family belongs to the cell surface receptors, which transduce intracellular signals in response to PAMPs (Anderson, 2000; Chow et al, 1999; Jiang et al, 2000; Kaisho and Akira, 2002). So far at least 10 TLRs (TLR1 to TLR10) have been identified. TLR2 and TLR4 are known to mediate inflammatory responses to bacterial components (Anderson, 2000; Chow et al, 1999; Jiang et al, 2000; Kaisho and Akira, 2002), ie, TLR2 is responsible for the recognition of LTA, whereas TLR4 recognizes LPS (Chow et al, 1999; Kaisho and Akira, 2002). In immunocompetent cells such as macrophages, the response to LPS is mediated by interaction with TLR4 in conjunction with TLR4 accessory protein MD-2 and CD14, and transduces intracellular signals followed by the activation of TLR-associated adapter protein, myeloid differentiation factor 88 (MyD88), leading to the activation of nuclear factor (NF)-kappaB and then to a synthesis of proinflammatory cytokines (Chow et al, 1999; Jiang et al, 2000).

Epithelial cells lining the luminal tracts are also known to recognize microbia and their constituents by a set of receptors, referred to as pattern-recognition receptors. TLRs are known to function as the major epithelial pattern-recognition receptors in recognizing PAMPs. For example, intestinal epithelial cells (IECs) are continuously exposed to bacteria and their components, and are shown to be involved in innate immunity and to possess the TLR system (Cario and Podolsky, 2000; Hausmann et al, 2002; Hugot et al, 2001; Podolsky, 2002; Schilling et al, 2001).

The intrahepatic biliary tree is a conduit of bile, and biliary epithelial cells (BECs) serve as a critical barrier to bile (Nakanuma et al, 1997). There have been several reports that bacterial components such as LPS, LTA, and bacterial DNA fragments are detectable in normal and pathologic bile (Hiramatsu et al, 2000; Osnes et al, 1997; Sasatomi et al, 1998), suggesting that BECs are exposed to bacterial components. Recently, BECs were shown to secrete polymeric IgA and also lactoferrin into bile, thereby contributing to the biliary mucosal defense (Saito and Nakanuma, 1992; Sugiura and Nakanuma, 1989). Our previous

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study showed up-regulation of TNF-alpha in cultured murine BECs by LPS treatment (Zen et al, 2002). So far, the mechanisms by which the BECs regulate the inflammatory and innate immune responses of the biliary tree to bacterial components in bile remain unexplored with respect to TLR.

In this study, we investigated the expression of TLR and related molecules in cultured murine BEC and human intrahepatic cholangiocarcinoma (CC) cell lines. We also examined the responsiveness of these cells against bacterial components and the intracellular signaling.

Results

Detection and Measurement of LPS (Endotoxin) in Human Gallbladder Bile

By dot bolt analysis, LPS was detectable in two of the seven (29%) bile samples of gastric cancer and three of the five (60%) cholecystolithiasis bile samples (Fig. 1). By the limulus amebocyte lysate test, endotoxin (LPS) was measurable in four of the seven (57%) bile samples of gastric cancer and in four of the five (80%) cholecystolithiasis bile. Progressive dilution of the LPS sample produced a straight standard line. The average LPS level in bile samples was 349 ng per 1 mg protein (range, 1 to 1665; 236 $\pm$ 394 in normal bile and 462 $\pm$ 804 in cholecystolithiasis bile).

Detection of TLRs and Related Molecules in Cultured Murine BEC and Two CC Cell Lines

Reverse Transcription and PCR (RT-PCR) Study: Using RNA extracts from cultured murine BEC, amplification of TLR2 to TLR5, MyD88, MD-2, and CD14 mRNA was detected (Fig. 2). Using RNA extracts from cultured CC cells of CCKS1 (Saito et al, 1993; Sugawara et al, 1998) and HuCCTl (Miyagiwa et al, 1989), mRNA expression profile of TLR and related molecules were similar to those in murine BEC, though MyD88 in CCKSl was rather faintly detected. Neither TLR9, RP105, nor MD-1 mRNA was detectable in murine BEC or CC lines (Fig. 2).

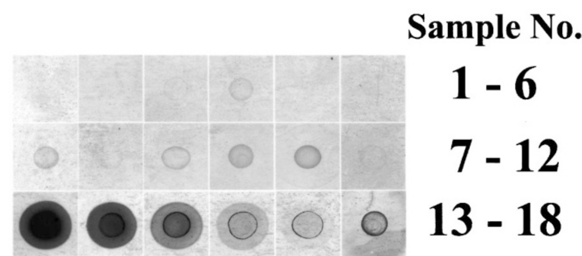

Figure 1.
Dot blot analysis for lipopolysaccharide (LPS). LPS samples extracted from 10 μl of gallbladder bile were directly blotted on membrane and immunodetected using anti-LPS polyclonal antibody. LPS was detected in two of seven (29%, samples No. 4 and 7) cases of gastric cancer (normal bile, samples No. 1 to 7) and three of five (60%, samples No. 9 to 11) cases of cholecystolithiasis (samples No. 8 to 12). The LPS sample extracted from *Escherichia coli* (sample No. 18) and commercially purchased LPS (sample No. 13 [5 μg], No. 14 [1 μg], No. 15 [200 ng], No. 16 [40 ng], No. 17 [8 ng]) were used as a positive control.

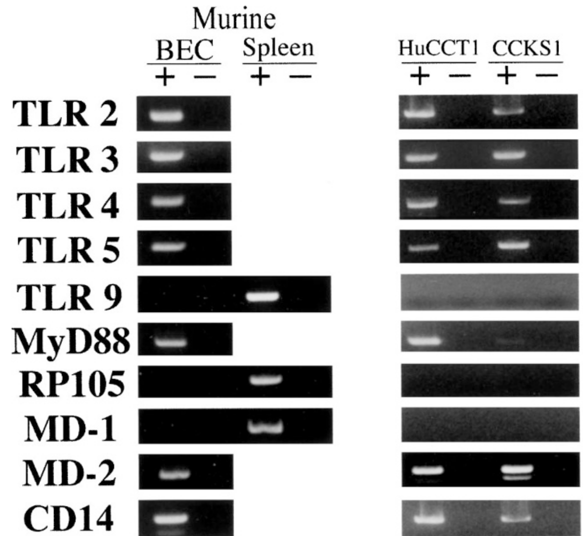

Figure 2.
Reverse transcription-PCR (RT-PCR) analysis of Toll-like receptors (TLRs) and their related molecules in cultured murine biliary epithelial cells (BECs), and in cultured human intrahepatic cholangiocarcinoma (CC) cell lines, CCKSI and HuCCTI. TLR2, 3, 4, and 5, MyD88, MD-2, and CD14 mRNAs were detected in cultured murine BECs, while TLR9, RP105, and MD-1 were not (murine spleen tissue was used as a positive control for TLR9, RP105, and MD-1). In cultured HuCCTI and CCKSI cell lines, TLR2, 3, 4, and 5, MyD88, MD-2, and CD14 mRNAs were distinctly found except for MyD88 in CCKSI (but three more cycles of PCR enabled a clear visualization). Each RT-PCR product gives bands of the appropriate molecular weight. + = addition of reverse transcriptase; − = addition of distilled water (negative control). PCR cycles were 3 to 5 more cycles than those shown in Table 1.

Immunohistochemical Study: TLR2 (Fig. 3A) and TLR4 (Fig. 3C) were expressed on the cell surface and also to a lesser degree in the cytoplasm of cultured cells. The expression of TLR3 (Fig. 3B), TLR5 (Fig. 3D), and MyD88 (Fig. 3E) was found in the cytoplasm as well as cell surface. CD14 was expressed in the cytoplasm and cell surface of CC cells (Fig. 3F), and its expression was prominent on the cell surface of murine BEC (Fig. 3H). Figure 3G is the negative control.

Production of TNF-Alpha in Cultured Murine BEC and CC Cell Lines after LPS, LTA, and Escherichia coli Treatment and Blocking Studies

TNF-Alpha Production after LPS, LTA, and E. coli Treatment: Treatment with LPS, LTA, and autoclaved and ultraviolet (UV)-treated *E. coli* up-regulated TNF-alpha mRNA expression in murine BEC and two CC lines (Fig. 4). Semi-quantitative assessment revealed that the average level of TNF-alpha mRNA was increased approximately 3- to 11-fold, compared with the control. The increase in TNF-alpha caused by LPS and autoclaved *E. coli* was significant when compared with that induced by UV-treated *E. coli* or LTA ($p < 0.05$).

ELISA revealed that two CC lines and murine BEC incubating with progressively diluted concentration of LPS or LTA secreted dose-dependently increasing amounts of TNF-alpha (Fig. 5).

Blocking Study by Anti-TLR4 Antibody: Preincubation with anti-TLR4 neutralizing antibody inhibited the

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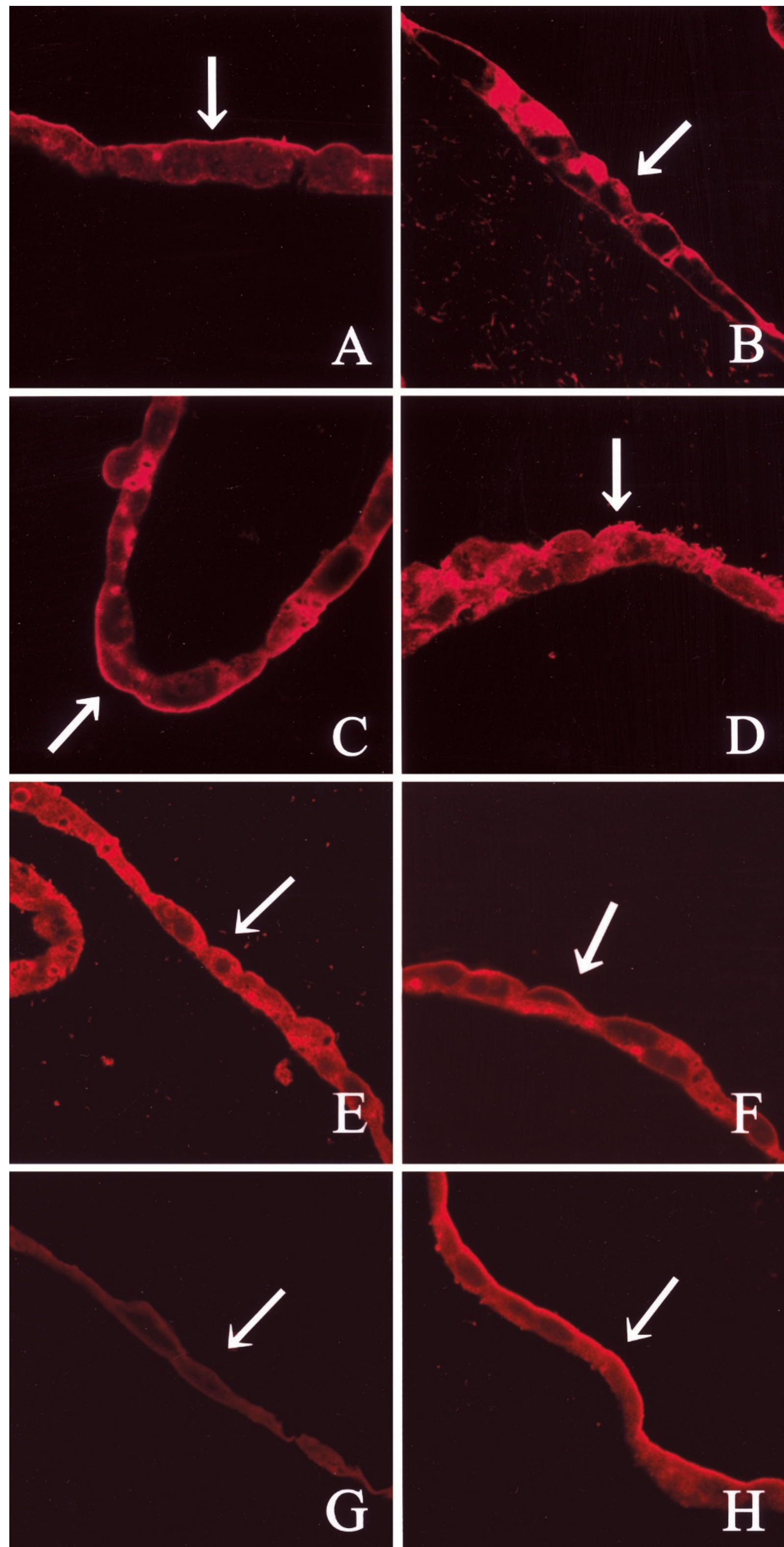

Figure 3.
Immunohistochemical detection of Toll-like receptors (TLR) and related molecules (TLR2, TLR3, TLR4, TLR5, MyD88, and CD14) in the human intrahepatic cholangiocarcinoma (CC) cells (HuCCT1) and CD14 in murine biliary epithelial cells (BECs) cultured on collagen gel. TLR2 (A, HuCCT1) and TLR4 (C, HuCCT1) is expressed on the cell surface membrane and also to a lesser degree in the cytoplasm of cultured cells. The expression of TLR3 (B, HuCCT1), TLR5 (D, HuCCT1), and MyD88 (E, HuCCT1) are found in the cytoplasm as well as cell surface membrane. CD14 is expressed in the cytoplasm and cell surface membrane of human CC cell (F, HuCCT1), but in murine BEC (H), its expression is prominent in the cell surface membrane. These positive signals are eliminated when the slide was incubated with normal rabbit IgG (negative control, G). *Arrows* show the surface of the cultured cells on collagen gels. Examination was done under a confocal laser scanning microscope.

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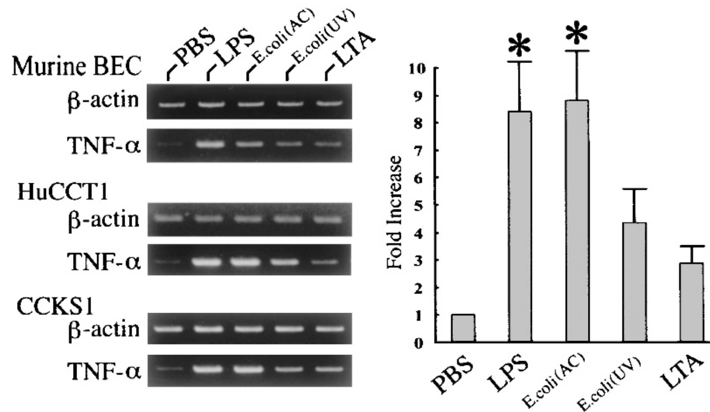

Figure 4.

Induction of TNF-alpha by lipopolysaccharide (LPS) (1 μg/ml), lipoteichoic acid (LTA) (10 μg/ml), autoclave-killed *Escherichia coli* (*E. coli (AC)*, 10 μg/ml, wet weight), and ultraviolet (UV)-irradiation-killed *E. coli* (*E. coli (UV)*, 10 μg/ml, wet weight) in culture cell lines. Cultured murine biliary epithelial cells (murine BEC), and cultured human cholangiocarcinoma (CC) cell lines (HuCCTI and CCKSI), were treated with or without these stimulants for 3 hours. TNF-alpha mRNA expression levels were determined by RT-PCR. The gel image of the PCR amplicon distinctly shows an increase in TNF-alpha production caused by all these stimulants to various degrees (representative gel image at left). The intensity was measured with the NIH Image Program, and the fold-increase in TNF-alpha production for each stimulant is expressed as a relative ratio with or without stimulant. Consequently, the average level of TNF-alpha induction from two independent experiments is approximately 3- to 9-fold, compared with no stimulant (PBS). Among these stimulants, the fold-increase in TNF-alpha production by LPS and autoclaved *E. coli* is significant compared with that of others (< 0.05). *Bars* denote SED.

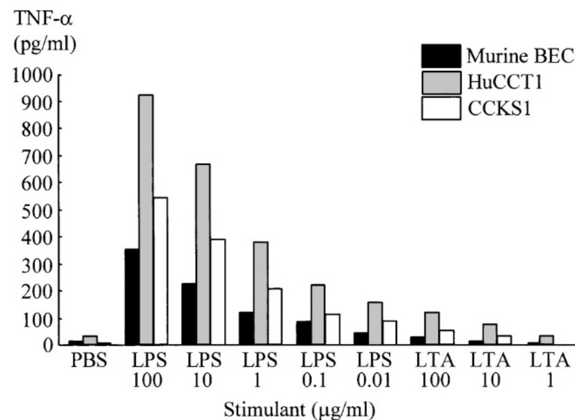

Figure 5.

Human intrahepatic cholangiocarcinoma (CC) cell lines (HuCCT1 and CCKS1) and murine biliary epithelial cells (BEC), which are subconfluent, were stimulated with 0.01 to 100 μg/ml lipopolysaccharide (LPS), 1 to 100 μg/ml lipoteichoic acid (LTA) or PBS (no stimulant) and cultured for 12 hours prior to assessment of TNF-alpha content in the supernatant by ELISA. A dose-response curve demonstrates that these cells secrete increased amounts of TNF-alpha according to the dose of LPS or LTA. However, the effect on TNF-alpha production by LTA is mild compared with that by LPS at the same concentration. Representative data of two experiments are shown.

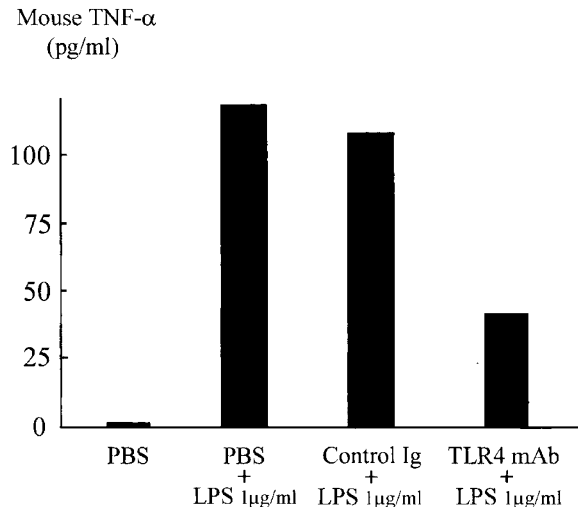

Figure 6.

Murine biliary epithelial cells were co-incubated with antimouse Toll-like receptor (TLR)4/MD-2 neutralizing antibody (clone MTS510), control isotype Ig, or PBS (vehicle control), and cultured with lipopolysaccharide (LPS) (1 μg/ml) for 12 hours prior to assessment of the supernatant for TNF-alpha content by ELISA. Spontaneous TNF-alpha secretion (PBS) was 3.4 $\pm$ 2.2pg/ml (mean $\pm$ SD of two values). TNF-alpha secretion in response to LPS alone (PBS + LPS) was 129 $\pm$ 16 pg/ml, control isotype Ig followed by LPS (control Ig + LPS) was 115 $\pm$ 11 pg/ml, and TLR4/MD-2 neutralizing antibody followed by LPS (TLR4 mAb + LPS) was 41 $\pm$ 10 pg/ml. The production of TNF-alpha is clearly but partially inhibited by TLR4 mAb. *Bars* denote SD of two values.

production of TNF-alpha in cultured murine BEC after LPS treatment (inhibitory rate was 32%) (Fig. 6). In contrast, the addition of isotype control antibody had no distinct inhibitory effect on TNF-alpha production. Inhibition by TLR4 antibody was not complete because TNF-alpha levels of murine BEC treated with LPS and anti-TLR4 antibody were higher than those of control cells without LPS treatment.

LPS Binding to the Surface of Cultured Human CC Cells

LPS conjugated to Alexa Fluor 488 was detected on the cultured cells, particularly at their cell periphery, as a honey-comb appearance (Fig. 7).

Activation of NF-Kappa B after LPS Treatment and Blocking Studies

Activation of NF-KappaB: Immunofluorescence staining showed the translocation of p65, a subunit of NF-kappaB, into the nucleus of two CC lines with treatment of 1 μg/ml LPS, though it remained in the cytoplasm of those cells without such treatment (Fig. 8). As for the DNA-binding capacity of NF-kappaB

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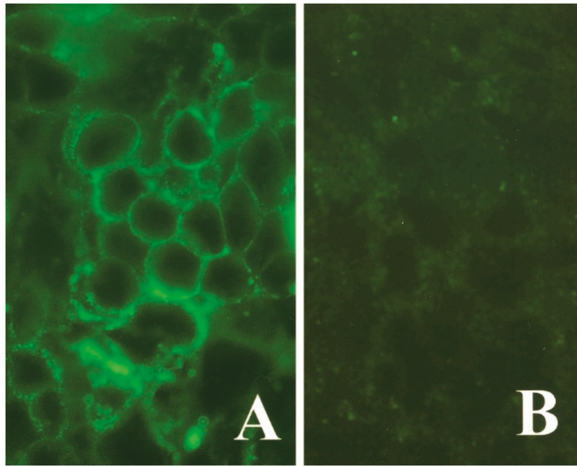

Figure 7.
Human intrahepatic cholangiocarcinoma cell lines (HuCCTl) cultured on LabTek Chamber slides were incubated with the LPS conjugated to Alexa Fluor 488. Positive fluorescence suggesting the presence of LPS is detected at the surface, with clear accentuation on the cell periphery of cultured cells (A). (B) is a negative control using Alexa Fluor 488 alone. The specimens were observed under a fluorescent microscope.

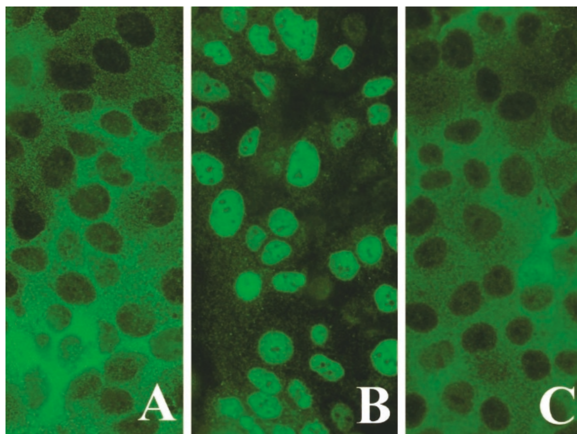

Figure 8.
Lipopolysaccharide (LPS)-induced nuclear factor (NF)-kappaB translocation into nuclei of the cultured human intrahepatic cholangiocarcinoma cell line, HuCCTl. HuCCTl cells grown on LabTek chamber slides were exposed to 1 μg/ml LPS for 1 hour. The slides were fixed and then immunostained using polyclonal antibody against p65 of NF-kappaB. The expression of p65 was found in the nucleus of LPS-treated cells (B), but in the cytoplasm of untreated cells (A). Concomitant treatment with LPS and 10 μM MG132, an inhibitor of NF-kappaB, prevented NF-kappaB nuclear translocation (C). The specimens were examined under a fluorescence microscope.

using a sensitive multi-well colorimetric assay (Renard et al, 2001), NF-kappaB activation in cell extracts from LPS and autoclaved *E. coli*–treated HuCCTl cells was found to be increased approximately 2-fold compared with that in untreated cells (Fig. 9). Moreover, these DNA-NF-kappaB binding activities were effectively competed by the wild-type consensus oligonucleotide but not the mutated one (Fig. 9), demonstrating specific NF-kappaB binding to its consensus oligonucleotide sequences.

Blocking Studies using NF-KappaB and Mitogen-Activated Protein Kinase (MAPK) Inhibitors: Pretreatment with MG132 (inhibitor of NF-kappaB) prevented nuclear translocation (Fig. 8) and DNA-binding activity (Fig. 9) of NF-kappaB, and significantly blocked the TNF-alpha mRNA synthesis in two CC lines (Fig. 10). Other inhibitors for MAPK (PD98059, SB203580, SP600125, U0126, and DMSO) failed to show such inhibition.

Discussion

In the gastrointestinal tract, TLR expressed in the IEC is a key transmembranous receptor with cytoplasmic signal domain to recognize microbes and their components and may be involved in the innate immunity and the development of enterocolitis by producing inflammatory molecules (Cario and Podolsky, 2000; Hausmann et al, 2002; Hugot et al, 2001; Podolsky, 2002). Similar processes using TLR may be operative in the biliary tree. In ascending cholangitis, pathogenic bacteria and their components in bile may elicit acute inflammation and induce secretion of proinflammatory cytokines by BEC (Liu et al, 1998; Matsumoto et al, 1994; Osnes et al, 1997; Yasoshima et al, 1998). It was shown in the current study that LPS or endotoxin was detected, and not infrequently, in normal gallbladder and cholecystolithiasis bile. These data suggest that BECs of the intrahepatic biliary tree are exposed to bacterial components in bile. Furthermore, Sasatomi et al (1998) reported that in primary biliary cirrhosis and primary sclerosing cholangitis, endotoxin accumulates abnormally in BECs of the intrahepatic bile ducts, suggesting that biliary lesions of these diseases might be causally related to bacterial components in bile (Tsuneyama et al, 2001). However, there have been no data available regarding the roles of TLR in BECs in the pathogenesis of inflammatory biliary diseases.

It was shown in this study that TLRs and related molecules were detectable in cultured BECs. That is, cultured murine BECs and two CC lines expressed mRNAs of TLR2, 3, 4, and 5, MD-2 (extracellular helper molecule enabling TLR4 and TLR2 in response to bacterial components) (Shimazu et al, 1999), MyD88 (an adaptor molecule essential for intracellular signaling via TLR/IL-1beta receptor family) (Takeuchi et al, 2000), and CD14. Immunohistochemically, TLR2, 3, 4, 5, and MyD88 and CD14 were detected on the apical and lateral surfaces of two CC lines. It seems reasonable that BECs may react to bacterial components via the activation of TLR2, 3, 4, and 5 as speculated in macrophages and IECs (Cario and Podolsky, 2000; Faure et al, 2000; Podolsky, 2002). As for the RP105, MD-1, and TLR9, cultured murine BECs and two CC lines failed to express their mRNAs. It has already been reported that expression of the RP105 and MD-1 complex is restricted to B cells (Nagai et al, 2002), which is compatible with our present data.

TNF-alpha is an inflammatory cytokine produced through TLR activation to bacterial components (Xu et al, 2001). This study showed that LPS and to a lesser extent LTA treatment induced an enhanced production of TNF-alpha in cultured murine BECs and two CC cell lines. ELISA analysis revealed that two CC cell

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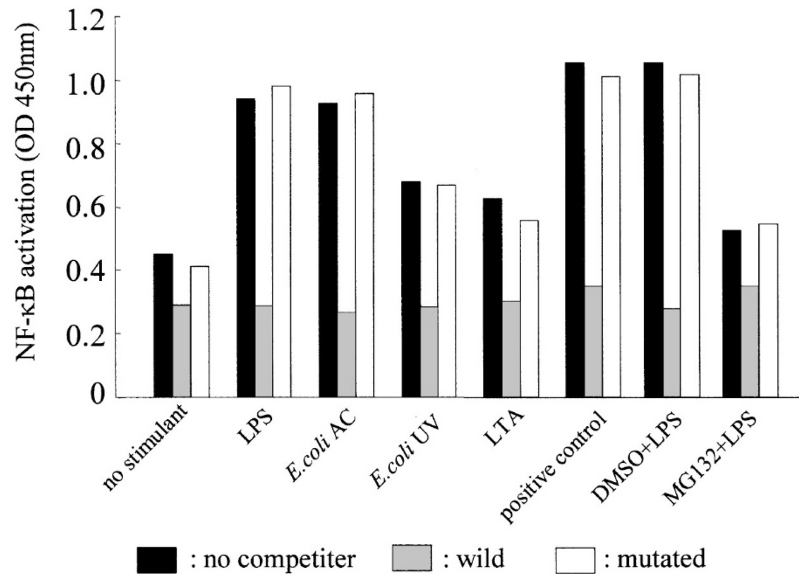

Figure 9.

Nuclear factor (NF)-kappaB and DNA binding assay of lipopolysaccharide (LPS)-induced NF-kappaB activation in the cultured human intrahepatic cholangiocarcinoma cell line, HuCCTI. Six groups from the left: HuCCTI cells were grown and incubated with LPS (1 μg/ml), lipoteichoic acid (LTA) (10 μg/ml), autoclave-killed *Escherichia coli* (*E. coli AC*, 10 μg/ml, wet weight), and UV-irradiation-killed *E. coli* (*E. coli UV*, 10 μg/ml, wet weight) for 3 hours, and then cell extracts were subjected to NF-kappaB and DNA binding assay. DNA-binding capacities of NF-kappaB with a consensus NF-kappaB–binding oligonucleotide in the extracts of LPS and autoclaved *E. coli*–treated cells are increased approximately 2-fold compared with those of untreated cells (no stimulant), while those of LTA and UV irradiated *E. coli*–treated cells are increased approximately 1.5-fold. TNF-alpha–treated HeLA cells were used as a positive control. Moreover, each DNA-NF-kappaB–binding activity is effectively competed for by the wild-type consensus oligonucleotide but not mutated oligonucleotide. Two groups from the right: HuCCTI cells were pretreated with 20 μM MG132 (NF-kappaB inhibitor) or DMSO (vehicle control) for 1 hour and then treated with 1 μg/ml LPS for 3 hours. Concomitant treatment with MG132 caused a significant decrease of NF-kappaB activation, demonstrating that 20 μM MG132 is sufficient to block the activation.

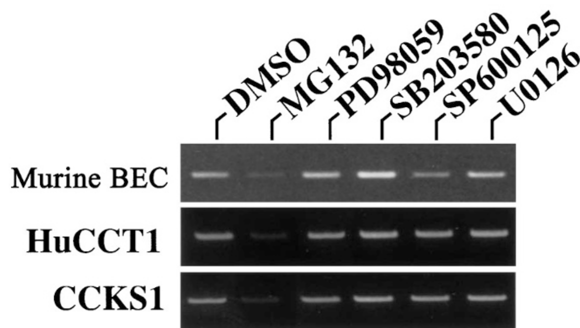

Figure 10.

Effect of nuclear factor (NF)-kappaB inhibitor and mitogen-activated protein kinase (MAPK) inhibitors on TNF-alpha production. TNF-alpha induction by LPS was inhibited by MG132 but not other inhibitors, demonstrating that NF-kappaB is critical for TNF-alpha induction. Cultured murine biliary epithelial cells and human cholangiocarcinoma cell lines, HuCCTI and CCKS1, were pretreated with 10 μM MG132 (an inhibitor of NF-kappaB), 100 μM PD98059 (an inhibitor of MEK1/2 in the p44/p42 MAPK pathway), 100 μM SB203580 (an inhibitor of the p38 MAPK pathway), 100 μM SP600125 (an inhibitor of tyrosine phosphorylation in the JNK MAPK pathway), U0126 (an inhibitor of MEK1/2 in the p44/p42 MAPK pathway), or DMSO (vehicle control) for 1 hour, and then treated with 1 μg/ml LPS for 3 hours. Total RNA was extracted and analyzed by RT-PCR with a specific primer for TNF-alpha.

lines and murine BECs incubated with different concentrations of LPS or LTA dose-dependently secreted TNF-alpha. Interestingly, the level of TNF-alpha production by autoclaved *E. coli* was higher than that of UV-irradiated *E. coli*. Autoclaved treatment is known to reduce the capsular material outside the membrane containing LPS of Gram-negative bacteria, but UV-irradiation treatment maintains capsular antigens (Aucken et al, 1998), suggesting that LPS is an effective molecule for recognition of Gram-negative bacteria by TLR. Taken together, bacterial components including fragmentous Gram-negative bacteria in bile may be at least partly involved in the pathophysiology of the intrahepatic biliary tree through TLR activation.

There have been several studies on the LPS-induced TLR activation and intracellular signaling in IECs (Cario and Podolsky, 2000; Hausmann et al, 2002; Hugot et al, 2001; Podolsky, 2002). Recently, TLR4 and MD-2 complex were shown to be the key components of LPS response machinery of IECs. Jiang et al (2000) reported that a molecular proximity between CD14 and TLR4, and LPS/CD14 complexes physically associate with TLR4 before the nuclear translocation of NF-kappaB. It was found in this study that in cultured BEC after LPS treatment, CD14 was first found to be expressed on cell surfaces. LPS was shown to bind to the cell surface of cultured CC cells as a honey-comb appearance, suggesting binding of LPS to CD14 on their cell surfaces. Thus, LPS/CD14 may elicit the intracellular signalings, though CD14 lacks transmembrane and intracellular domains. TLR family with transmembrane proteins with cell surface receptors and an intracytoplasmic signal domain may be more importantly involved in this intracellular signaling. It seems plausible that the recognition of LPS by TLR with CD14 lead to the reaction with the intracellular adaptor protein MyD88 and MD-2 and that CD14 and TLR4 with MD-2 and MyD88 work

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cooperatively in response to LPS treatment in cultured BECs as speculated in other cell lineages. In fact, cultured murine BECs and two CC cell lines expressed MD-2 and also MyD88 at mRNA levels in addition to TLR4 and CD14. Their proteins were also detected immunohistochemically. The blocking of TLR4 by anti-TLR4 neutralizing antibody inhibited the production of TNF-alpha after LPS treatment, confirming that the effect of LPS on BECs was at least partly mediated by TLR4 (Faure et al, 2000). The incomplete inhibition by the TLR4 antibody may be due to the involvement of other receptors such as TLR2 in LPS-mediated signaling in cultured BECs (Yang et al, 1998, 1999).

Activation of the TLR family is known to result in the translocation and activation of NF-kappaB followed by the synthesis of proinflammatory cytokines required for the immune responses in macrophages and IECs (Cario and Podolsky, 2000; Faure et al, 2000; Hausmann et al, 2002; Podolsky, 2002). Such issues have not been examined in BECs. NF-kappaB is a transcription factor that, under basal conditions, is sequestered as an inactive form in the cytoplasm through its interaction with inhibitory proteins (I-kappaB). Stimuli lead to the phosphorylation and subsequent proteasome-mediated degradation of I-kappaB, followed by the translocation of NF-kappaB to the nucleus, where it binds to a specific sequence (5'-GGGACTTTCC-3') in the promoter region of genes to activate their transcription (Ghosh et al, 1998). It was shown in this study that LPS treatment induced a translocation of NF-kappaB into the nucleus. Such a translocation of NF-kappaB after TLR activation is already known to occur in monocytes and endothelial cells.

Furthermore, increased DNA binding activity of NF-kappaB was shown by the assessment of the DNA-binding capacity of NF-kappaB with a consensus NF-kappaB–binding oligonucleotide in CC cells. These results suggest that LPS can induce the translocation and increased DNA binding activity of NF-kappaB in cultured CC cells. More importantly, concomitant treatment with MG132, an inhibitor of NF-kappaB, significantly prevented the induction of TNF-alpha by LPS treatment as well as the NF-kappaB nuclear translocation and the increased NF-kappaB-DNA binding, confirming that production of TNF-alpha is mediated in a NF-kappaB–dependent manner in these cells. Another intracellular signaling pathway responsive to LPS treatment, the IL-6-mediated activation of the p44/p42 MAPK pathway (eg, MEK1 and 2), was reported in human immortalized BECs (Park et al, 1999). However, this pathway was not operative in the production of TNF-alpha by LPS treatment in the cultured cells in this study because neither of the MAPK inhibitors affected the production of TNF-alpha.

In conclusion, the present study showed first that cultured BECs expressed TLR and related molecules. LPS bound directly to the surface of these cells and probably formed the receptor complex of CD14, TLR4, and MD-2 in an association with MyD88 in BECs, mediating the activation of NF-kappaB and synthesis of proinflammatory cytokines (TNF-alpha). Although the present study is limited to cultured cells, these findings suggest that the TLR family in BECs of the intrahepatic biliary tree work as a PAMP recognition system and contribute to the innate immunity and immunopathologic processes in vivo. More study is needed to clarify the pathogenesis of several inflammatory biliary diseases, such as primary biliary cirrhosis and primary sclerosing cholangitis.

Materials and Methods

Cell Culture of Murine BECs and CC Cell Lines

Murine intrahepatic BECs were isolated, purified, and cultured from 8-week-old female BALB/C mice (Charles Liver Japan, Yokohama, Japan) as described previously (Katayanagi et al, 1998, 1999). Manipulations of these mice were done according to the guidelines for the care and use of laboratory animals at the Takaramachi Campus of Kanazawa University. Purified BECs were incubated with a culture medium composed of Dulbecco's modified Eagle medium (D-MEM) and Nutrient Mixture F-12, 1:1, (Gibco, Rockville, Maryland), 10% ν-Serum (Becton Dickinson, Bedford, Massachusetts), 1% ITS+ (Becton Dickinson), 5 μM Forskolin (Wako Pure Chemical, Osaka, Japan), 12.5 mg/ml of Bovine pituitary extract (Gibco), 1 μM dexamethasone (Sigma Chemical, St Louis, Missouri), 5 μM triiodo-thyronine (Sigma Chemical), 5 mg/ml of glucose (Sigma), 25 mM sodium bicarbonate (Sigma Chemical), and 25 ng/ml of mouse epidermal growth factor (Gibco) at 37° C in an atmosphere of 5% CO_2 for 2 weeks. The passage of cultured BECs was achieved using collagenase solution composed of 0.2 g of collagenase S-l (Nittazeratin, Osaka, Japan) and 1.082 g of dispase (Gibco) in 500 m1 of D-MEM/F-12 (Gibco). Eleventh subcultured BECs were used for the study.

Two CC cell lines, CCKS1 (Saito et al, 1993; Sugawara et al, 1998) and HuCCTl (JCRB0425) (Miyagiwa et al, 1989), were also used. CCKS1 was established in our laboratory and HuCCTl was purchased from Health Science Research Resources Bank (Osaka, Japan).

Determination of Baseline Expression of TLR by RT-PCR

Two CC lines were cultured in culture flasks with a standard medium, RPMI 1640 for HuCCTl (GIBCO) and D-MEM/F12 for CCKS1, supplemented with 10% FCS and murine BECs on collagen-coated culture plates overlaid with the above-mentioned culture medium, respectively, at 37° C in a water-saturated atmosphere of 95% air and 5% CO_2 for 48 hours. Cultured cells were collected from culture flasks or plates with cell scraper or collagenase treatment (Sigma) for determination of baseline mRNA expression of TLR2, 3, 4, 5, and 9 and of MyD88, RP105, MD-1 and -2, and CD14 by RT-PCR. Murine spleen tissue was used as a positive control for TLR9, RP105, and MD-1 (Nagai et al, 2002). Briefly, total RNA was isolated from each sample with the RNeasy Total RNA System (Qiagen, Hilden, Germany) and treated with RNase-

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free DNaseI. For RT, 1 μg of total RNA, M-MLV RTase (ReverTra Ace, Toyobo, Tokyo, Japan) and oligo-dT primers were used. PCR amplification was performed using DNA polymerase (Takara EX *Taq*, Takara, Tokyo, Japan) and specific primers for murine or human mRNA sequences (Table 1). The primers for β-actin mRNA were used as a house-keeping gene. Following PCR, an annealing of primers for 1 minute and an extension at 72° C for 2 minutes (the annealing temperature and cycle number are shown in Table 1), PCR products were subjected to agarose gel electrophoresis.

Immunohistochemistry for TLR and Related Molecules

Two CC lines and murine BECs cultured on collagen gel for 2 days were fixed in 10% neutral buffered formalin and embedded in paraffin. Then, more than 10 thin sections were cut from each paraffin block. Deparaffinized sections of CC lines were incubated at 4° C overnight with primary polyclonal antibodies against human TLR2 (1 μg/ml), human TLR3 (1 μg/ml), human TLR4 (1 μg/ml), human TLR5 (1 μg/ml), and human MyD88 (1 μg/ml). Deparaffinized sections of CC lines and of murine BECs were incubated with primary polyclonal antibody against mouse CD14 (also reacts with human CD14, 1 μg/ml). All of these primary antibodies were rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, California). Antibodies for immunostaining of MD-2 were not available. Then, these sections were treated with goat anti-rabbit immunoglobulins conjugated to alkaline phosphatase labeled polymer (Simple Staining Kit, Nichirei, Tokyo, Japan) for 1 hour. As a negative control, normal rabbit IgG (1 μg/ml) was used for primary antibody. The specimens were observed under confocal laser scanning microscopy (LSM5, Carl Zeiss Japan, Tokyo, Japan).

Examination of LPS-Binding to Cultured Cells

Two CC lines grown on LabTek chamber slide for 2 days were incubated with LPS conjugated to Alexa Fluor 488 (10 μg/ml, Molecular Probes, Eugene, Oregon) for 1 hour, and the specimens were observed under a fluorescent microscope.

Measurement of NF-kappaB Activation

DNA-Binding Capacity of NF-kappaB: This was assayed by a sensitive multi-well colorimetric assay

Table 1. Primer Sequences Used in this Study

	Forward	Reverse	PCR product	Annealing	Cycle No.[a]
Murine					
TLR2	5′-TGGAGACGCCAGCTCTGGCTCA-3′	5′-CAGCTTAAAGGGCGGGTCAGAG-3′	380bp	60°C	22
TLR3	5′-GGTGGTCCCGTTAATTTCCT-3′	5′-CAGGAGCATACTGGTGCTGA-3′	489bp	60°C	22
TLR4	5′-AGTGGGTCAAGGAACAGAAGCA-3′	5′-CTTTACCAGCTCATTTCTCACC-3′	311bp	60°C	23
TLR5	5′-TCTCTTGGAACTTCGGCTGT-3′	5′-AGAAGATAAAGCCGTGCGAA-3′	545bp	60°C	27
TLR9	5′-GCAAGCTCAACCTGTCCTTC-3′	5′-CAGGCTAAGACACTGGAGGC-3′	503bp	57°C	28
MyD88	5′-CACTCGCAGTTTGTTGGATG-3′	5′-CGCAGGATACTGGGAAAGTC-3′	597bp	60°C	27
RP105	5′-GTCCCAGCAGAGGACCATTA-3′	5′-AGCCCAGCCCTAGGAACTAA-3′	399bp	57°C	38
MD-1	5′-CAGTGTTCCAAGCAGATCCA-3′	5′-GCAGGCAAGGAGTAGCTTTG-3′	605bp	57°C	38
MD-2	5′-GAAGCAACAGTGGTTCTGCAA-3′	5′-GCGGTGAATGATGGTGAAAT-3′	412bp	60°C	22
CD14	5′-CTGATCTCAGCCCTCTGTCC-3′	5′-GCAAAGCCAGAGTTCCTGAC-3′	454bp	60°C	22
TNF-alpha	5′-GGCAGGTCTACTTTGGAGTCATTGC-3′	5′-ACATTCGAGGCTCCAGTGAATTCGG-3′	307bp	60°C	22
beta-actin	5′-GCTATGCTCTCCCTCACGCCAT-3′	5′-ACGCAGCTCAGTAACAGTCCGC-3′	648bp	60°C	15
Human					
TLR2	5′-GCCAAAGTCTTGATTGATTGG-3′	5′-TTGAAGTTCTCCAGCTCCTG-3′	347bp	55°C	23
TLR3	5′-CCATTCCAGCCTCTTCGTAA-3′	5′-GGATGTTGGTATGGGTCTCG-3′	505bp	55°C	23
TLR4	5′-TGGATACGTTTCCTTATAAG-3′	5′-GAAATGGAGGCACCCCTTC-3′	507bp	55°C	28
TLR5	5′-GGAACCAGCTCCTAGCTCCT-3′	5′-GATGGCATCCTGGATATTGG-3′	575bp	55°C	23
TLR9	5′-CGTGACAATTACCTGGCCTT-3′	5′-GTCCTGTGCAAAGATGCTGA-3′	435bp	55°C	34
MyD88	5′-CCAACCTTCAGCAGTGACAA-3′	5′-GTGTGTATGCTGGTGCCTGT-3′	398bp	55°C	23
RP105	5′-CCATGCTCAAGGGACTCTGT-3′	5′-GTTTGCAAGTGGGACAGGTT-3′	403bp	55°C	34
MD-1	5′-GGGCCTGTCAATAATCCTGA-3′	5′-GGAGGCGACCAATTAGAGAA-3′	399bp	55°C	34
MD-2	5′-TCCACCCTGTTTTCTTCCAT-3′	5′-GGGCTCCCAGAAATAGCTTC-3′	404bp	55°C	24
CD14	5′-GGTTCCTGCTCAGCTACTGG-3′	5′-CTTGGCTGGCAGTCCTTTAG-3′	594bp	55°C	27
TNF-alpha	5′-GCTGTACCTCATCTACTCCCA-3′	5′-GCAATTTCTAGGTGAGGTCTTC-3′	660bp	55°C	21
beta-actin	5′-CAAGAGATGGCCACGGCTGCT-3′	5′-TCCTTCTGCATCCTGTCGGCA-3′	275bp	60°C	18

[a] Shows a standard number of PCR cycles in stimulatory and inhibitory studies.

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(Renard et al, 2001) using a TransAM NF-kappaB Kit (Active Motif, Carlsbad, California). Briefly, cultured murine BECs on culture plates and two CC lines in culture flasks were scraped and centrifuged for 10 minutes at 1000 rpm. The pellet was resuspended in 100 μl of lysis buffer containing a protease inhibitor cocktail, and the lysate was centrifuged for 20 minutes at 14,000 rpm. Supernatant constitutes the total protein extract, and the protein content was measured using a Bradford-based assay (Nippon Rio-Rad, Tokyo, Japan). Cell extracts (5 μg) from each sample and also TNF-α–treated HeLa cells (positive control), were incubated in 96-well plates coated with NF-kappaB consensus double-stranded oligonucleotide sequence (5'- AGTTGAGGGGACTTTCCCAGGC-3') for 1 hour and then with supplied primary anti–NF-kappaB antibody (1:1000) for 1 hour, and subsequently with secondary peroxidase-conjugated antibody (1:1000) for 1 hour. After a colorimetric reaction, the optical density was read at 450 nm. Competition experiments were conducted with the 22-bp double-strand DNA, either wild-type (see above) or mutated: 5'-AGTTGAGCTCACTTTCCCAGGC (with three mutated bases underlined).

Immunohistochemistry for NF-KappaB: Two CC lines grown on a LabTek chamber slide (Nalge Nunc, Naperville, Illinois) were divided into two groups: one was treated with LPS, and the other was not treated. The former was further divided into two groups: one was treated with 1 μg/ml of LPS for 1 hour, and the other with 1 μg/ml of LPS and an inhibitor of NF-kappaB, MG132. These cultured cells were fixed in 4% paraformaldehyde and permeabilized with cold acetone, and then reacted with polyclonal antibody against p65 of NF-kappaB (rabbit IgG, 5 μg/ml, IBL, Fujioka, Japan) and Alexa Fluor 488 goat fluorescent anti-rabbit IgG antibody (diluted 1:1000, Molecular Probes). The specimens were observed under a fluorescent microscope.

Detection and Measurement of Endotoxin (LPS) in the Gallbladder Bile

Gallbladder bile was aseptically collected during laparotomy or laparoscopic cholecystotomy in 12 patients (seven men and five women) aged 43 to 71 years (median, 56), including seven patients with gastric cancer (used as a normal bile) and five with cholecystolithiasis. All bile samples were stored in sterilized tubes at $-80°$ C.

LPS Extraction and Dot Blotting: LPS was extracted from bile samples using the phenol-water extraction method (Westphal and Jann, 1965), with an LPS extraction kit (Intron Biotechnology, Kyungki-Do, Korea). Briefly, 100-μl volumes of bile samples were suspended in 1.2 ml of lysis buffer for 5 minutes. The suspension was then centrifuged at 6700 $\times g$ for 10 minutes at 4° C, and LPS fraction (aqueous phase) was collected. LPS was sedimented after centrifugation and dried. LPS was resuspended in 100 μl of pyrogen-free water and 10 μl of them (corresponding to 10 μl of original bile) was applied to a nitrocellulose membrane. The membrane was first incubated for 1 hour with rabbit polyclonal anti-LPS antibody (1:100, East Coast Biologics, North Berwick, Maine), and for 1 hour with goat anti-rabbit immunoglobulins conjugated to peroxidase labeled-dextran polymer (Envision, diluted 1:10, Dako Japan, Kyoto, Japan) and visualized with a benzidine reaction. As positive controls, *E. coli* and the graded concentrations of commercially purchased LPS (from *E. coli* serotype 055:B5, Sigma) were used.

Protein Extraction and LPS Assay: Bile samples were homogenized in protein lysis solution (tissue protein extraction reagent, Pierce, Rockford, Illinois). After centrifugation at 13,000 $\times g$ for 10 minutes, the supernatant was transferred to a fresh tube; the total concentration of protein in the lysate was measured by a Bradford-based assay. A volume corresponding to 200 μg of protein was diluted to a volume of 3 ml in pyrogen-free water and assayed by a limulus amebocyte lysate test (Endotoxin ES, Mitsubishi Kagaku, Tokyo, Japan) (Osnes et al, 1992). Furthermore, to confirm that LPS liquated out in the lysis solution, *E. coli* (see below) and LPS (Sigma) were used as positive controls, and LPS volume (ng/1 mg protein) was measured from a standard curve on which were plotted the graded concentrations of LPS. As a negative control, pyrogen-free water was used.

Intracellular Signalings via TLR Activation after LPS, E. coli, and LTA Treatment

TNF-alpha production at mRNA level was used as a marker of TLR activation and intracellular signaling (Akashi et al, 2000; Xu et al, 2001).

Preparation of E. coli, LPS, LTA, and Inhibitors of NF-KappaB and of MAPK: A strain of nonpathogenic *E. coli*, which was biochemically identical to the standard strain of *E. coli*, ATCC25922 (American Type Culture Collection, Rockville, Maryland), was isolated from a clinical sample and identified using a multimedia tube for the identification of enterobacteria (Enterotube II, Becton Dickinson). This *E. coli* grown in Luria-Bertani medium (LB medium, Wako Pure Chemical) was collected at the exponential growth phase at an OD of 0.18, and then was completely killed by autoclave or UV irradiation and used for the following studies. LPS (from *E. coli* serotype 055:B5, Sigma), LTA (from *Streptococcus faecalis*, Sigma), the proteasome inhibitor MG132 (Calbiochem, San Diego, California) as an inhibitor of NF-kappaB, and PD98059 (Calbiochem), SB203580 (Calbiochem), SP600125 (Biomol Research Laboratories, Plymouth Meeting, Pennsylvania), and U0126 (Calbiochem) as an inhibitor of the MAPK signaling pathway were purchased commercially.

Production of TNF-Alpha in Cultured Cells after LPS, E. coli, and LTA Treatment: Murine BECs and two CC lines grown to semiconfluence in plates were treated with LPS (1 μg/ml), autoclaved *E. coli* (1 μg/ml, wet weight), UV-irradiated *E. coli* (1 μg/ml, wet weight), or LTA (10 μg/ml) in fresh standard medium for 3 hours and were used for an isolation of RNA and

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RT-PCR. The intensity was semiquantitatively assessed the NIH Image Program. As a control, PBS (PBS) was added to the culture medium.

Two CC lines and murine BEC were untreated or incubated with a progressively diluted concentration of LPS (100 μg/ml to 0.01 μg/ml) or LTA (100 μg/ml to 1 μg/ml), and culture supernatants were assayed for human or murine TNF-alpha by ELISA (Biosource International, Camarillo, California).

Blocking by Anti-TLR4 Antibody: Cultured murine BECs were preincubated with monoclonal rat anti-mouse TLR4/MD-2 neutralizing antibody (clone MTS510, TLR4 function-blocking antibody, HyCult biotechnology, Uden, the Netherlands) (Akashi et al, 2000), or with isotype control IgG at a concentration of 10 μg/ml for 1 hour, and then incubated with 1 μg/ml LPS for 12 hours. Then, the mouse TNF-alpha in culture supernatants was measured by ELISA (Biosource International). As a control, cultured murine BECs without preincubation were treated with LPS for 12 hours.

Intracellular Signaling for TNF-Alpha Production after LPS Treatment and Inhibition Studies: Preliminary study showed that LPS treatment produced up-regulation of TNF-alpha in murine BECs and two CC lines. This signaling pathway was examined. The translocation of NF-kappaB into the nuclei after LPS treatment was evaluated immunohistochemically (see above). The activation of NF-kappaB was assessed by measuring NF-kappaB and DNA binding assay (see above). Then, to determine whether the activation of NF-kappaB or MAPK signaling by LPS is critical for the up-regulation of TNF-alpha, cultured murine BECs and two CC lines were pretreated with 10 μM MG132 (inhibitor of NF-kappaB), 100 μM PD98059 (inhibitor of MEK [MAP kinase] 1 and 2 in the p44/p42 MAPK pathway), 100 μM SB203580 (an inhibitor of the p38 MAPK pathway), 100 μM SP600125 (inhibitor of the JNK MAPK pathway), 100 μM U0126 (inhibitor of MEK1/2 in the p44/p42 MAPK pathway), or dimethyl sufoxide (vehicle control) for 1 hour, and then treated with 1 μg/ml of LPS for 3 hours. Then, they were washed three times before isolation of RNA and RT-PCR study. TNF-alpha mRNA level was measured in each culture assay.

Statistical Analysis

The Student's t test was employed with a level of significance of $p < 0.05$.

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