Lipopolysaccharide Induces Overexpression of MUC2 and MUC5AC in Cultured Biliary Epithelial Cells

Possible Key Phenomenon of Hepatolithiasis

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Bacterial infection, bile stasis, mucin hypersecretion, and an alteration of the mucin profile such as an aberrant expression of gel-forming apomucin (MUC2 and MUC5AC) in the intrahepatic biliary tree are thought to be important in the lithogenesis of hepatolithiasis. So far, there have been no detailed studies linking bacterial infection to altered mucus secretion of biliary epithelium. In this study, the influence of lipopolysaccharide (LPS), a bacterial component, on apomucin expression in cultured murine biliary epithelial cells was examined with emphasis on the participation of tumor necrosis factor (TNF)- α . It was found that LPS up-regulated the expression of MUC2 and MUC5AC in cultured murine biliary epithelial cells. LPS also induced the expression of TNF- α in biliary epithelial cells and its secretion into the culture medium. The up-regulation of these apomucins was inhibited by pretreatment with TNF- α antibody. TNF- α alone also induced the overexpression of MUC2 and MUC5AC in cultured biliary epithelial cells. This overexpression was inhibited by pretreatment with calphostin C, an inhibitor of protein kinase C. These findings suggest that LPS can induce overexpression of MUC2 and MUC5AC in biliary epithelial cells via synthesis of TNF- α and activation of protein kinase C. This mechanism might be involved in the lithogenesis of hepatolithiasis. (*Am J Pathol* 2002, 161:1475–1484)

Brown pigment stones are major calculi in hepatolithiasis, and mucin is known to be an integral component of such stones.^{1,2} Recent studies disclosed that epithelial mucins have in common a protein backbone, called apomucin or mucin core protein (MUC).^{3,4} To date, 10 types of apomucins have been identified and they show tissue- or cell-specific expression or distribution in the human body.^{5–8} In the biliary tract, the expressions of 6 apomucins (MUC1, MUC2, MUC3, MUC5AC, MUC5B, and MUC6) have been reported.⁹ The hypersecretion of mu-

cin and alteration of the mucin profile in the intrahepatic biliary tree may relate to the development of biliary diseases including hepatolithiasis.¹ For example, the up-regulation of MUC2, MUC3, MUC5AC, MUC5B, and MUC6 expression was shown in stone-containing intrahepatic bile ducts,^{1,10} and the aberrant and enhanced expression of gel-forming mucins (MUC2 and MUC5AC) in the intrahepatic biliary tree may play an important role in the process of stone formation.¹

Bacterial infection and bile stagnation are also thought to be crucial for the lithogenesis of hepatolithiasis.^{11,12} *Escherichia coli* (*E. coli*), *Klebsiella*, *Streptococcus*, and *Pseudomonas* are the most frequent isolates from the bile in hepatolithiasis cases.¹¹ β -glucuronidase from gram-negative bacteria, particularly *E. coli*, may be involved in the deconjugation of bilirubin, a process followed by the formation of calcium-bilirubinate, a major component of brown pigment stones.

The relation between bacterial infection and overexpression of MUC2 and MUC5AC was emphasized especially in the respiratory tract and middle ear.^{13–16} Bacterial components or tumor necrosis factor- α (TNF- α), a proinflammatory cytokine, is able to induce overexpression of MUC2 and MUC5AC in airway and middle ear epithelial cells or goblet cells.^{13–20} Furthermore, it is reported that protein kinase C (PKC) is involved in these processes.¹⁸ However, the exact relationship between bacterial infection and aberrant expression of MUC2 and MUC5AC in biliary tree has yet to be examined.

In this study, we tried to evaluate the relationship between bacterial infection, the hypersecretion of mucin, especially MUC2 and MUC5AC, and the participation of TNF- α in the lithogenetic process using murine-derived cultured biliary epithelial cells (BECs).

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Materials and Methods

Cell Culture of Murine Intrahepatic BECs

Basic Culture and Passage

Murine intrahepatic BECs were isolated from 8-week-old female BALB/c mice and were purified and cultured, as described previously.^{21,22} The purified BECs were seeded at a density of $1 \times 10^5/\text{cm}^2$ on a collagen-coated dish, and were incubated with a culture medium composed of D-MDM/F-12 (Dulbecco's modified Eagle medium and nutrient mixture F-12, 1:1; Life Technologies, Inc., Rockville, MD), 10% Nu-Serum (Becton Dickinson Labware, Bedford, MA), 1% ITS+ (Becton Dickinson Labware), 5 $\mu\text{mol/L}$ forskolin (Wako Pure Chemical Industries, Ltd., Osaka, Japan), 12.5 mg/ml of bovine pituitary extract (Life Technologies, Inc.), 1 $\mu\text{mol/L}$ dexamethasone (Sigma Chemical Co., St Louis, MO), 5 $\mu\text{mol/L}$ triiodothyronine (Sigma Chemical Co.), 5 mg/ml of glucose (Sigma Chemical Co.), 25 mmol/L sodium bicarbonate (Sigma Chemical Co.), 1% antibiotics-antimycotic (Life Technologies, Inc.), and 25 ng/ml of mouse epidermal growth factor (Life Technologies, Inc.) at 37°C in an atmosphere of 5% CO_2 for 2 weeks. The cells formed a monolayer with a sheet-like growth pattern. The passage of cultured BECs was made using collagenase solution composed of 0.2 g of collagenase S-1 (Nittazzeratin, Osaka, Japan) and 1.082 g dispase (Life Technologies, Inc.) in 0.5 l of D-MEM/F-12 (Life Technologies, Inc.) followed by incubation with the above-mentioned basic culture medium. Seventh subcultured BECs were used in the experiments.

Cell Culture with Treatment of Lipopolysaccharide (LPS), TNF- α , Anti-TNF- α Antibody, and PKC Inhibitor

Cultured BECs were incubated with a bacterial component, LPS, or TNF- α to determine whether either could change apomucin gene and protein expression. Furthermore, cultured BECs were preincubated with TNF- α antibody or PKC inhibitor to clarify whether they influence the change in apomucin gene and protein expression induced by LPS.

LPS Treatment: The BECs cultured to semiconfluence were treated with 100 $\mu\text{g/ml}$ of LPS (from *Escherichia coli*, serotype O55:B5; Sigma Chemical Co.). The concentration was based on previous studies using cultured gallbladder epithelium.^{23,24} LPS levels in human bile that had a positive culture for gram-negative bacteria showed great individual variation ranging from 140 pg/ml to 27.8 $\mu\text{g/ml}$, and the local LPS concentration at the site of infection is thought to exceed this range.²⁵ Our preliminary data showed that lactate dehydrogenase activity was not elevated in the culture medium after addition of LPS at this concentration (100 $\mu\text{g/ml}$), implying no significant leakage of lactate dehydrogenase from the cultured cells. That is, at this concentration, LPS would not induce the direct necrosis of BECs. This was consistent with a previous report.²³

TNF- α Treatment: The BECs cultured to semiconfluence were treated with 0.1, 1.0, and 10.0 ng/ml of murine TNF- α (recombinant) (R&D System Inc., Minneapolis, MN). This concentration of TNF- α was based on previous report.²³ Lactate dehydrogenase activity was not elevated in the culture medium after the addition, implying that TNF- α would not induce direct cell-necrosis at these concentrations.

TNF- α Blocking: For the blocking of TNF- α , cultured BECs were preincubated for 1 hour with 10 $\mu\text{g/ml}$ of monoclonal antibody against mouse recombinant TNF- α (XT22; Endogen, Woburn, MA) before addition of LPS to the culture medium. This concentration of anti-TNF- α antibody was based on a previous report.²³

PKC Inhibition: For inhibition of PKC, cultured BECs were preincubated for 1 hour with 0.1 $\mu\text{mol/L}$ of calphostin C, a PKC inhibitor (Sigma Chemical Co.), before addition of LPS and TNF- α to the culture medium. This concentration of calphostin C was decided on by referring to a previous report.²⁶

Cell Preparation for Experiments

The BECs incubated with LPS for 6, 12, 24, and 48 hours were used for the reverse transcriptase-polymerase chain reaction (RT-PCR) of TNF- α , interleukin (IL)-1 α , IL-1 β , and IL-6, and interferon- γ mRNA expression, and the culture medium was used for enzyme-linked immunosorbent assay (ELISA) of TNF- α . The expression of apomucin was examined at the mRNA level in BECs incubated with LPS alone or LPS plus anti-TNF- α antibody for 12 hours (RT-PCR), and at the protein level in those incubated with LPS alone or LPS plus anti-TNF- α antibody for 24 hours (Western blot). BECs incubated with LPS alone, LPS plus anti-TNF- α antibody, LPS plus calphostin C, TNF- α alone, and TNF- α plus calphostin C were used for PKC activity assays (incubation for 4 hours), and for Northern blot analyses of apomucin gene expression (incubation for 12 hours). In each experiment, BECs incubated with basic medium were used as a control. The expression of CD14 and TNF receptors (CD120a, CD120b) at the protein and mRNA level in BECs incubated with basic medium was similarly examined by RT-PCR and Western blotting.

Expression of Apomucins, Cytokines, CD14, and TNF Receptors (mRNA Level)

RNA Extraction

Total RNA was isolated from ~ 80 mg of cells (1×10^6) by the guanidinium thiocyanate-phenol-chloroform method, using Isogen reagent (Wako Pure Chemical Industries, Ltd.). After that, RNA was dissolved in 50 μl of distilled water containing 0.1% diethylpyrocabonate and quantitated, using a spectrophotometer at OD²⁶⁰. Isolated RNA was used for the following RT-PCR and Northern blot analyses.

Table 1. Sequences and Annealing Temperature, Cycle Times, and Product Size of PCR Primers

	Forward/reverse	Sequence	Annealing temperature (°C)	Cycle time	Product size (bp)
MUC1	Forward	ccccagttgtctgttggggtc	65	25	205
	Reverse	ggattctaccaccacggagcc			
MUC2	Forward	acgatgcctacaccaaggctc	60	41	210
	Reverse	tgatcttctgcatgttccca			
MUC3	Forward	aaagttgtcacactggggc	64	42	210
	Reverse	ccaagtcctacacacaggct			
MUC5AC	Forward	ggaccaagtgggttgacactgac	50	35	257
	Reverse	cctcatagttgaggcacatccag			
TNF- α	Forward	ggcaggctctacttggagtcattgc	60	30	307
	Reverse	acattcgaggctccagtgattcgg			
IL-1 α	Forward	tggccaaagttcctgactgtttg	58	37	488
	Reverse	caggctatttaaccaagtggtgct			
IL-1 β	Forward	gaaatgccacctttgacag	54	34	504
	Reverse	caaggccacaggtattttgt			
IL-6	Forward	cgtggaaatgagaaaagagttgtgc	60	40	561
	Reverse	atgcttaggcataacgcactaggt			
INF- γ	Forward	actggcaaaaggatggtgac	60	35	273
	Reverse	tgagctcattgagatgcttg			
CD14	Forward	ctgatctcagccctctgtcc	60	35	453
	Reverse	gcaaagccagagttcctgac			
CD120a	Forward	gccggatatgggcatgaagc	55	37	200
	Reverse	tgtctcagccctcacttgac			
CD120b	Forward	tcatgccacctgtcagtgag	60	32	380
	Reverse	cgtggatattctccaggcac			
β -actin	Forward	gctatgctctccctcacgccat	60	25	626
	Reverse	acgcagctcagtaacagtcgcc			

RT-PCR

RT-PCRs for MUC1, MUC2, MUC3, MUC5AC, TNF- α , IL-1 α , IL-1 β , IL-6, INF- γ , CD14, CD120a, CD120b, and β -actin were performed as described previously.²⁷ The oligonucleotide sequences, numbers of cycles, and annealing temperatures of these primers are shown in Table 1. As quantitative controls, primers for the β -actin gene, a housekeeping gene, which is considered to be constitutively expressed, were used. After PCR, 5- μ l aliquots of the products were subjected to 1.5% or 2.0% agarose gel electrophoresis and stained with ethidium bromide. The density of the bands was analyzed using NIH image for semiquantification. The appropriate number of PCR cycles for semiquantitative analysis was established from a blotting curve of density of each PCR-amplified product. The ratio of the density of the target product to that of β -actin was calculated for each sample. Four samples were prepared for each PCR analysis.

Northern Blot Analysis

Northern blot analysis was performed by AlkPhos Direct (Amersham Pharmacia Biotech, Uppsala, Sweden).²⁸ Complementary DNA (cDNA) of MUC2 and MUC5AC mRNA was obtained by RT-PCR using RNA extracted from fresh mouse colon or stomach as template, respectively (MUC2: sense 5'-acgatgcctacaccaaggctc-3', antisense 5'-ccatgttattggggcatttc-3', annealing temperature 52°C, 40 cycles, product size 509 bp; MUC5AC: sense 5'-tcatgagtgcacacaggagc-3', antisense tgaccacagatcctcatctc-3', annealing temperature

55°C, 40 cycles, product size 683 bp). Alkaline phosphatase-labeled cDNA probes were obtained by incubating of each PCR product with reaction buffer, labeling reagent, and cross-linker for 30 minutes at 37°C. These three solutions were included in AlkPhos Direct. Forty μ g of total RNA were subjected to electrophoresis on 1.0% agarose gel in the presence of formaldehyde. RNA was transferred by capillary blotting onto an Hybond+ N nylon membrane (Amersham Pharmacia Biotech) and cross-linked with ultraviolet light using Spectrolinker (Tomy Digital Biology Co., Ltd., Tokyo, Japan). The membrane was incubated with the prehybridization buffer included in AlkPhos Direct for 30 minutes at 50°C. After that, the labeled probe was added to the prehybridization buffer at a concentration of 15 ng/ml, and then hybridized at 50°C for 18 hours. The membrane was washed twice with primary wash buffer [2 mol/L urea, 0.1% sodium dodecyl sulfate (SDS), 50 mmol/L sodium phosphate buffer, pH 7.0, 1 mmol/L NaCl, and 10 mmol/L MgCl₂] for 10 minutes at 50°C, and then twice more with secondary wash buffer (50 mmol/L Tris base and 100 mmol/L NaCl) for 5 minutes at room temperature. Signals were detected with CDP-Star chemiluminescent detection reagent (Amersham Pharmacia Biotech). The membrane was incubated with detection reagent (40 μ l/cm²) for 5 minutes at room temperature and then placed in a detection bag with Hyperfilm (Amersham Pharmacia Biotech) for 3 hours, for image development. After the detachment of probes by incubation with 5% SDS for 30 minutes at 50°C and 0.1% SDS for 5 minutes at 100°C, the membrane was reused.

Expression of Apomucins, Cytokines, CD14, and TNF Receptors (Protein Level)

Immunohistochemistry for MUC1, MUC3, and MUC5AC

Murine-derived BECs were cultured three-dimensionally in collagen gel²⁹ with or without LPS addition. After formalin fixation and paraffin embedding, the sections from the collagen gel matrix including cultured BECs were used for the immunohistochemistry, using Histofine Simple Stain MAX (Nichirei, Tokyo, Japan) for MUC1, the streptavidin-biotin peroxidase complex for MUC3, and the EnVision+ system (DAKO, Glostrup, Denmark) for MUC5AC. After the blocking of endogenous peroxidase, the deparaffinized sections were incubated overnight at 4°C with individual primary antibodies: anti-mouse MUC1 (sc-6828, 1:100, goat polyclonal; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-mouse MUC3 (MBEC3, 1:20, rabbit monoclonal; established in our laboratory)³⁰ and anti-human MUC5AC (45M1, 1:200, mouse monoclonal; Novocastra Laboratories Ltd., Newcastle, UK). Our preliminary study showed that the monoclonal antibody against human MUC5AC immunohistochemically cross-reacted with murine MUC5AC, because this antibody reacted with the mucous-secreting cells of the murine gastric mucosa in which MUC5AC is known to be abundant. Sections for MUC1 or MUC5AC were incubated with rabbit anti-goat immunoglobulins, or goat anti-mouse immunoglobulins, both of which were conjugated to peroxidase-labeled polymer [Histofine Simple Stain MAX PO(G), Envision+]. Sections for MUC3 were incubated with secondary biotinylated antibody, anti-rat immunoglobulin (1:100; Biosource International, Camarillo, CA), and then treated with the StreptABComplex (DAKO). 3,3'-Diaminobenzidine tetrahydrochloride was used as the chromogen, and the sections were counterstained with hematoxylin. Negative controls were evaluated by substituting the primary antibodies with nonimmunized serum.

Protein Extraction

Proteins were extracted from cultured BECs using T-PER Tissue Protein Extraction Reagent (Pierce Chemical Company, Rockford, IL). Total protein was measured by a spectrophotometer. Extracted protein was used for Western blot analysis and PKC activity assay. Fifty and 10 µg of protein was used as sample in the analysis and assay, respectively.

Western Blot Analysis

The analysis was performed on 4% SDS-polyacrylamide gel electrophoresis gel for MUC1, MUC3, and MUC5AC or 10% SDS-polyacrylamide gel electrophoresis gel for CD14, CD120a, and CD120b. The proteins in the gel were electrophoretically transferred onto nitrocellulose membrane. The membranes were incubated with primary antibodies to MUC1 (sc-6828, 1:500), MUC3

(MBEC3, 1:200), MUC5AC (45M1, 1:500) (same antibodies described in immunohistochemistry), CD14 (M-305, 1:50, rabbit polyclonal; Santa Cruz Biotechnology, Inc.), CD120a (H-271, 1:200, rabbit polyclonal; Santa Cruz Biotechnology, Inc.), and CD120b (L-20, 1:200, goat polyclonal; Santa Cruz Biotechnology, Inc.). Anti-mouse MUC2 antibody was not available for this study. MUC proteins expression was detected using a catalyzed signal amplification (CSA) system, peroxidase (DAKO). CD14, CD120a, and CD120b protein expression was detected using second antibodies conjugated to peroxidase-labeled polymer such as EnVision+ system (DAKO) for CD14 or CD120a and Histofine Simple Stain MAX PO (G) (Nichirei) for CD120b. 3,3'-Diaminobenzidine tetrahydrochloride was used as the chromogen.

ELISA of TNF-α

The TNF-α concentration in the culture medium was determined by ELISA using a mouse TNF-α ELISA kit (R&D Systems Inc). The quantity of TNF-α in the medium was interpolated from a mouse TNF-α antigen standard curve, which ranged from 23.4 pg/ml to 1.5 ng/ml. Four samples were prepared for each measurement.

PKC Activity Assay

PKC activity was determined using the Pk-Select Protein Kinase C assay kit (Exalpha Biologicals, Inc., Boston, MA). A reaction solution was made by mixing 15 µmol/L ATP solution, 25 µmol/L biotinylated PKC pseudosubstrate solution, activation solution (0.3 mg/ml of phosphatidylserine, 30 µg/ml of diacylglycerol, and 0.3% Triton X-100), and PKC reaction buffer (0.5 mmol/L calcium chloride, 10 mmol/L magnesium chloride, and 20 mmol/L Tris-HCl pH 7.5), with gamma ³²P-ATP. Samples (5 µl) were added to 20 µl of the above reaction mixture and incubated for 15 minutes at 30°C. Incubation was terminated by adding and mixing in 10 µl of stop solution (8.0 mol/L guanidine hydrochloride). Negative controls consisted of similar assay samples without substrate peptides. Eight µl of avidin solution was added to the terminated reaction samples. After an incubation of 5 minutes at room temperature, 50 µl of wash solution and 20 µl of the reaction sample were added sequentially into the sample reservoirs of centrifugal ultrafiltration units. After a spin of 5 minutes at 14,000 × g, washing procedure were performed three times by adding 100 µl of wash solution followed by a spin of 5 minutes at 14,000 × g. The sample reservoirs were transferred into units of scintillation vials and the vials examined with a channel set for ³²P.

Statistics

Values are given as the mean ± SEM. Means of two groups were compared with the Mann-Whitney *U*-test. Intergroup comparisons of three groups were done with Scheffé's test. Values of *P* < 0.05 were regarded as statistically significant.

Figure 1. Expression of CD14, CD120a, and CD120b in cultured murine BECs. **A:** RT-PCR revealed mRNA expression of CD14 (453 bp), CD120a (200 bp), and CD120b (380 bp). **B:** Western blot analysis revealed protein expression of CD14 (53 kd), CD120a (55 kd), and CD120b (75 kd).

Results

Expression of CD14, CD120a, and CD120b in Cultured BECs

The expression of CD14 and TNF receptors (CD120a and CD120b) was shown in BECs at the mRNA and protein level using RT-PCR and Western blot analysis (Figure 1).

Apomucin Gene and Protein Expression of BECs on LPS Treatment

MUC1, MUC2, MUC3, and MUC5AC mRNA were all expressed in control BECs incubated with basic medium as measured by RT-PCR. MUC2, MUC3, and MUC5AC mRNA expression was significantly enhanced by LPS treatment ($P < 0.05$) (Figure 2A). The up-regulation of MUC2 and MUC5AC mRNA expression was more prom-

inent. The expression levels of MUC2 and MUC5AC after LPS treatment were approximately four and five times higher than those of control BECs.

The blocking of TNF- α by anti-TNF- α antibody significantly inhibited the expression of MUC2 and MUC5AC mRNA. But the inhibition was not complete, because MUC2 and MUC5AC mRNA levels of BECs treated with LPS and TNF- α blocking were higher than those of control BECs ($P < 0.05$). TNF- α blocking was not evident in the up-regulation of MUC3 transcription induced by LPS. No apparent difference in MUC1 expression was observed among these three models. This result implied that the overexpression of MUC2 and MUC5AC mRNA induced by LPS was partially mediated via TNF- α , whereas the overexpression of MUC3 mRNA was not related to TNF- α (Figure 2B).

The results of Western blot analysis of MUC1, MUC3, and MUC5AC using protein extracted from BECs are shown in Figure 3. The bands of these proteins were broad in the high molecular weight region >200 kd. MUC1 protein expression was not changed by LPS treatment with or without TNF- α antibody. LPS enhanced MUC3 protein expression, although this expression was not inhibited by TNF- α antibody. LPS also enhanced MUC5AC protein expression, and this expression was inhibited by preincubation with TNF- α antibody, although MUC5AC protein levels in BECs treated with LPS and TNF- α antibody were slightly higher than in control BECs. These results were the same as those for mRNA evaluated by RT-PCR, confirming that the enhanced expression of MUC5AC caused by LPS was partially mediated via TNF- α , but that of MUC3 was not mediated via TNF- α .

Immunohistochemically, MUC1 was expressed at the cell membrane and also in the cytoplasm of scattered cultured BECs. MUC3 was expressed at the cell membrane of all BECs, particularly on the apical side of three-dimensionally cultured BECs. No apparent change was observed in MUC1 and MUC3 expression in cultured

Figure 2. **A:** RT-PCR of MUC1, MUC2, MUC3, and MUC5AC mRNAs in the cultured BECs. **B:** Expression levels of these apomucin mRNAs in BECs with or without LPS and TNF- α antibody were compared. No significant changes were observed among the three groups in MUC1. LPS significantly enhanced the expression of MUC2, MUC3, and MUC5AC. In BECs treated with LPS and TNF- α antibody, the LPS-induced expression of MUC2 and MUC5AC was partially inhibited, but MUC3 expression was not. The ratio of the band density of apomucin mRNA to β -actin mRNA was taken as the expression level. Results are expressed as a ratio of each expression level to the expression level of control BECs, and the data represent the mean $\pm$ SEM. β -actin was used as an internal marker. Control, BECs incubated with basic medium; LPS, BECs incubated with LPS alone; LPS + TNF- α , BECs incubated with LPS and TNF- α antibody; RELC, ratio to the expression level of control. *, $P < 0.05$.

Figure 3. Immunoblot analysis of MUC1, MUC3, and MUC5AC. Signals were detected in the high molecular weight region greater than 200 kD that consisted of mucin glycoproteins. Signals for MUC1 were almost of the same intensity in the three groups. MUC3 and MUC5AC were overproduced in the LPS-treated group. In BECs incubated with LPS and TNF- α antibody (TNF- α Ab), the signal for MUC3 was comparable to that of the LPS-treated group, and the signal for MUC5AC was weaker than that of the LPS-treated group. Control, BECs incubated with basic medium; LPS, BECs incubated with LPS alone; LPS + TNF- α Ab, BECs incubated with LPS and TNF- α antibody.

BECs with and without LPS. Although MUC5AC expression was not detected immunohistochemically in BECs without LPS treatment, it was seen in BECs after treatment with LPS.

Expression and Secretion of Cytokines Including TNF- α Induced by LPS Treatment

RT-PCR revealed that TNF- α , IL-1 α , and IL-1 β mRNA were constantly expressed in BECs cultured with basic medium (Figure 4A). IL-6 and INF- γ were not detected. Although IL-1 α and IL-1 β expression was weak, TNF- α expression was strong. Addition of LPS significantly enhanced the expression of TNF- α mRNA. This enhancement was most prominent at 6 hours after the addition of LPS and declined subsequently (Figure 4B), implying that LPS enhanced the expression of TNF- α mRNA transiently at an early phase. The expression of IL-1 α and

Figure 5. ELISA of TNF- α in the culture medium. TNF- α in the medium of BECs treated with LPS was significantly increased at 6 to 48 hours after addition of LPS compared to nontreated BECs. TNF- α was quantified using a mouse TNF- α antigen standard curve. Results represent the mean $\pm$ SEM. Control, the culture medium of nontreated BECs; LPS, the culture medium of LPS-treated BECs. *, $P < 0.05$ versus controls.

IL-1 β mRNA did not change after the addition of LPS (Figure 4A).

TNF- α quantified by ELISA in the culture medium of BECs treated with LPS was significantly increased at 6 hours compared to that of nontreated BECs, and TNF- α secretion had increased ~ 2.2 -fold at 48 hours (260.52 ± 21.35 pg/ml versus 120.31 ± 15.32 pg/ml) (Figure 5), implying that LPS enhanced TNF- α transcription and induced hypersecretion of TNF- α into the medium at 6 hours and thereafter.

Effect of PKC on MUC2 and MUC5AC mRNA Expression of BECs Induced by TNF- α or LPS Treatment

The results of Northern blot analysis for MUC2 and MUC5AC mRNA expression in BECs cultured with TNF- α , LPS, TNF- α antibody, or calphostin C, are shown in Figure 6. MUC2 mRNA was not detected in BECs cultured

Figure 4. **A:** RT-PCR of TNF- α , IL-1 α , and IL-1 β mRNAs in BECs with or without LPS treatment. BECs expressed these three inflammatory cytokines but the expression of IL-1 α and IL-1 β was weak compared to that of TNF- α . **B:** Time-course of TNF- α mRNA expression in BECs cultured with or without LPS. TNF- α expression was significantly increased by addition of LPS in the early phase. The ratio of the band density of cytokine mRNA to β -actin mRNA was taken as the expression level, and the expression level at time 0 (just before the addition of LPS) was regarded as the expression level of control. Results are expressed as a ratio of each expression level to the expression level of the control and the data represent the mean $\pm$ SEM. β -actin was used as an internal marker. RELC, ratio to the expression level of control. Control, BECs incubated with basic medium; LPS, BECs incubated with LPS. *, $P < 0.05$ versus controls.

Figure 6. Northern blot analysis of MUC2 (A) and MUC5AC (B) mRNA expression in BECs cultured with LPS, TNF- α , TNF- α antibody, and calphostin C. A and B: In BECs cultured with TNF- α (1.0 ng/ml and 10.0 ng/ml), signals for MUC2 and MUC5AC mRNA were detected, and their levels showed a dose-dependent manner. In BECs cultured with TNF- α and calphostin C, MUC2 showed no signal and MUC5AC a very weak signal. In BECs cultured with LPS, signals for MUC2 and MUC5AC were detected. In BECs cultured with LPS and TNF- α antibody or calphostin C, no band of MUC2 and MUC5AC was observed. Signals for MUC2 or MUC5AC mRNA were confirmed using RNA extracted from murine colon or stomach. Lane 1, control; lane 2, TNF- α (0.1 ng/ml); lane 3, TNF- α (1.0 ng/ml); lane 4, TNF- α (10.0 ng/ml); lane 5, TNF- α (10.0 ng/ml) and calphostin C; lane 6, LPS (100 μ g/ml); lane 7, LPS (100 μ g/ml) and TNF- α antibody; lane 8, LPS (100 μ g/ml) and calphostin C; lane 9, stomach; lane 10, colon. C: In ethidium bromide-stained gel, RNA was applied in each lane is approximately equal amounts.

with basic medium. MUC2 mRNA was detected in BECs cultured with TNF- α at a concentration of 1.0 ng/ml or 10.0 ng/ml, while it was not observed at 0.1 ng/ml. The signal for MUC2 mRNA in BECs cultured with TNF- α (10.0 ng/ml) was more intense than that in BECs treated with 1.0 ng/ml of TNF- α . These findings suggested that the MUC2 expression induced by TNF- α was dose-dependent. The expression was inhibited by preincubation with calphostin C, suggesting that TNF- α induced the mRNA expression via activation of PKC. Signal for MUC2 mRNA was detected in BECs cultured with LPS, although no signal was observed in BECs treated with LPS and TNF- α antibody or with LPS and calphostin C. These results indicated that LPS induced MUC2 mRNA expression via production of TNF- α and via activation of PKC.

The results of the mRNA expression for MUC5AC were similar to those for MUC2 (Figure 6B). Signal for MUC5AC mRNA was not detected in the BECs cultured with basic medium. Signals were detected in BECs cultured with TNF- α at a concentration of 1.0 ng/ml and 10.0 ng/ml, the

Figure 7. PKC assay of cultured BECs. In BECs incubated with TNF- α , PKC was significantly activated compared to control BECs, and in a dose-dependent manner. In BECs cultured with TNF- α and calphostin C, TNF- α -induced activation of PKC was inhibited. In BECs cultured with LPS, PKC was significantly activated, which was inhibited in BECs treated with LPS and TNF- α antibody or LPS and calphostin C. Control, BECs incubated with basic medium; TNF, BECs incubated with TNF- α ; TNF + CP, BECs incubated with TNF- α and calphostin C; LPS, BECs incubated with LPS; LPS + TNF-Ab, BECs incubated with LPS and TNF- α antibody; LPS + CP, BECs incubated with LPS and calphostin C; *, $P < 0.05$ versus control; **, $P < 0.05$ versus TNF (10.0 ng/ml); ***, $P < 0.05$ versus LPS.

signal for the latter being the more intense. The MUC5AC mRNA expression induced by TNF- α treatment was inhibited by preincubation with calphostin C. These results implied that TNF- α induced MUC5AC mRNA expression in a dose-dependent manner and also via activation of PKC. Signal for MUC5AC was detected in BECs cultured with LPS, and disappeared on preincubation with TNF- α antibody and also with calphostin C, suggesting that LPS induced MUC5AC mRNA expression via production of TNF- α and activation of PKC.

Alteration of PCK Activity in BECs Caused by LPS and TNF- α Treatment

PKC activities of BECs cultured with TNF- α alone, TNF- α plus calphostin C, LPS alone, LPS plus TNF- α antibody, LPS plus calphostin C, and basic medium, are shown in Figure 7. The PKC activity in BECs cultured with TNF- α (0.1 ng/ml, 1.0 ng/ml, and 10.0 ng/ml) was 29.2 ± 1.2 , 45.3 ± 4.3 , and 89.9 ± 5.3 pmol/minute/mg, respectively. These values were all significantly higher than the levels in control BECs (20.1 ± 0.8 pmol/minute/mg) ($P = 0.02$). This result implied that TNF- α treatment activated PKC in a dose-dependent manner. The activation by TNF- α (10.0 ng/ml) was completely inhibited by preincubation with calphostin C, a PKC inhibitor. The PKC activity of BECs cultured with LPS (100 μ g/ml) was 41.4 ± 3.6 pmol/minute/mg, which was significant compared to that of control BECs ($P = 0.02$). The activation of PKC induced by LPS was completely inhibited by preincubation with TNF- α antibody ($P = 0.02$), implying that LPS activated PKC via TNF- α . Preincubation with calphostin C also resulted in a complete inhibition of the activation of PKC induced by LPS ($P = 0.02$).

Figure 8. Mechanism of MUC2 and MUC5AC overproduction and secretion in BECs induced by LPS. LPS binds to CD14 expressed on BECs, which is followed by TNF- α synthesis and secretion. Secreted TNF- α reacts with TNF receptors (CD120a and CD120b) in an autocrine or paracrine manner. TNF- α activates PKC, which mediates the up-regulation of MUC2 and MUC5AC transcription and the secretion.

Discussion

The main findings obtained in this study using cultured murine-derived BECs can be summarized as follows: 1) LPS induced an enhanced expression of MUC2 and MUC5AC in BECs. 2) LPS induced the up-regulation of production of TNF- α in BECs, and TNF- α induced the overexpression of MUC2 and MUC5AC in BECs. 3) Furthermore, overexpression of MUC2 and MUC5AC induced by LPS or TNF- α was mediated by activation of PKC in BECs. 4) Cultured murine-derived BECs expressed CD14, and TNF receptors (CD120a and CD120b). Based on these main findings, the relationship of LPS, CD14, TNF- α , TNF receptors, PKC, and MUC2 and MUC5AC secretion is shown schematically in Figure 8.

In hepatolithiasis, bacterial infection via the biliary tract is thought to be important in lithogenesis.^{11,12} It was found in this study that LPS, a gram-negative bacterial component, induced enhanced expression of MUC2 and MUC5AC in cultured BECs. In addition, CD14, a receptor of LPS,³¹ was expressed in cultured BECs. Thus, it seems possible that LPS could directly bind to biliary epithelium via CD14, *in vivo*, and then might be involved in biliary epithelial expression of MUC2 and MUC5AC. Although CD14 was first described as a monocyte/macrophage differentiation antigen,³² it is also expressed in some epithelial cells.³³

The overexpression of MUC2 and MUC5AC induced by LPS was inhibited by pretreatment with anti-TNF- α antibody, implying that LPS enhanced the expression of MUC2 and MUC5AC via TNF- α . Interestingly, the treatment of BECs with LPS resulted in the overexpression of TNF- α and its secretion into the culture medium. The participation of TNF- α in the overproduction of MUC2 and MUC5AC was more directly shown by stimulation of BECs with TNF- α alone. Furthermore, TNF receptors (CD120a and CD120b)^{34,35} were also expressed in BECs, implying not only that TNF- α produced by cultured BECs could react to BECs in an autocrine or paracrine

manner *in vitro*, but also that TNF- α produced by inflammatory cells or adjacent BECs could react to BECs in hepatolithiasis *in vivo*. Our results were similar to that of cultural studies of goblet cells and human airway epithelial cells,^{17–19} suggesting that a common signal pathway could function in these cultured epithelial cells. On the other hand, overexpression of MUC3 induced by LPS was not inhibited by pretreatment with anti-TNF- α antibody.

The aberrant expression of MUC2 and MUC5AC is thought to be very important to the lithogenetic process, because these apomucins have the ability to form large polymers.^{1,10} When the proportion of MUC2 and MUC5AC in biliary mucin increases, the mucus in the biliary tract becomes more viscous, and MUC2 and MUC5AC could participate in both the nuclear formation of stones and their enlargement.^{1,10,36} In this context, MUC2 and MUC5AC could become not only an initiator, but also a promoter.

The relationship between bacterial infection and overexpression of MUC2 and MUC5AC has been evaluated in the respiratory tract and middle ear.^{13–16} Overproduction of MUC2 and MUC5AC is important in the pathogenesis of cystic fibrosis, bronchial asthma, or otitis media with effusion, and TNF- α and PKC are involved in this process of overexpression.^{13–20} Taken together, a common signal pathway could function in the epithelial cells of the respiratory tract, middle ear, and intrahepatic biliary tract.

Interestingly, the MUC2 and MUC5AC gene and protein expression induced by LPS and by TNF- α showed similar patterns. However, MUC1 and MUC3 differed in their expression. Both the MUC2 and MUC5AC genes are located on chromosome 11 at p15.5 in a 400-kd region.³⁷ This region also contains the genes for MUC5B and MUC6.³⁷ These four gene products (MUC2, MUC5AC, MUC5B, and MUC6) show considerable sequence homology, particularly in their cysteine-rich carboxyl-terminal regions, and their genes might have evolved from a common ancestor.³⁷ However, these four genes are unlikely to be completely controlled in the same manner. For example, phorbol 12-myristate 13-acetate (PMA) can induce overexpression of MUC2 and MUC5AC, although it has much less of an effect on the expression of MUC5B or MUC6 in a human colonic tumor cell line.³⁸ However, restricted to only two genes (MUC2 and MUC5AC), PMA, LPS, TNF- α , gram-positive and gram-negative bacteria are all able to up-regulate the transcriptions of both genes simultaneously.^{14,15,38,39} Furthermore, the up-regulation of MUC2 and MUC5AC expression by PMA or TNF- α is known to be mediated by PKC.^{18,38–40} Therefore, it is likely that although the promoters of MUC2 and MUC5AC differ substantially in sequence, they seem to be functionally homologous with respect to their sensitivity to at least some intracellular signals.¹⁶

It would be interesting to know the kinds of signal cascades or transcription factors involved in the occurrence of MUC2 and MUC5AC induced by LPS or TNF- α in cultured BECs. There are some key sequences in the MUC2 gene promoter.^{15,16,41} The κ B-binding site exists upstream of the MUC2 gene, which can bind to a transcription factor, nuclear factor (NF)- κ B.^{15,16} MUC2 tran

scription induced by *P. aeruginosa* was mediated by the binding of NF- κ B (p55 and p65 subunits) to this κ B site.^{15,16} Indeed, TNF- α can induce or activate several transcription factors including NF- κ B.^{42–44} The induction of NF- κ B expression by TNF- α is known to be mediated in a PKC-dependent or PKC-independent manner.^{45–48} In this study, MUC2 and MUC5AC expression induced by TNF- α was mediated via the activation of PKC, where NF- κ B might have participated as one of the transcription factors. Furthermore, several GC boxes are also present in the MUC2 promoter, which can be a binding site for the specificity protein (Sp) family of transcription factors including Sp-1.⁴¹ This Sp-1 is another candidate involved in the TNF- α -induced expression of MUC2 and MUC5AC.⁴¹ Signaling pathways between TNF- α or PKC and Sp-1 were suggested in various cell lines such as human glioma cells or human hepatoma cells.^{49,50} Although these pathways have not been determined in cultured BECs, Sp-1 may be involved in the process of TNF- α -induced MUC2 and MUC5AC expression in BECs.

In the present study, the overexpression of MUC2 and MUC5AC induced by LPS was partially inhibited by the blocking of TNF- α , and MUC2 and MUC5AC expression in BECs treated with LPS plus TNF- α antibody was enhanced compared to that in BECs cultured with basic medium (Figures 2 and 3). That is, preincubation with anti-TNF- α antibody could not totally inhibit the expression of MUC2 and MUC5AC induced by LPS, suggesting that the overexpression of MUC2 and MUC5AC caused by LPS was partially mediated by signaling other than a TNF- α -dependent pathway. An exact comparison between the MUC2 and MUC5AC expression of control BECs and that of BECs treated with LPS plus TNF- α antibody could not be made by Northern blot analysis (Figure 6), because no signal for MUC2 or MUC5AC mRNA was obtained in either BEC. According to previous reports on respiratory tract and colon epithelial cells,^{13–16} production of mucin induced by gram-positive or gram-negative bacteria is dependent on tyrosine kinase such as the MEK1/2-MAPK signaling pathway. LPS is known to activate a tyrosine kinase pathway in human monocytes and vascular endothelial cells, part of which is mediated in a TNF- α -independent manner.^{51–53} A MEK1/2-MAPK signaling pathway also constitutively exists in immortalized human cholangiocytes, and LPS activates this pathway in those cells.⁵⁴ Furthermore, this tyrosine kinase signal results in the activation of NF- κ B in respiratory tract epithelial cells, which are involved in the overproduction of mucin induced by *P. aeruginosa*.¹⁵ These tyrosine kinases and the continuous activation of NF- κ B may also be involved in the overexpression of MUC2 and MUC5AC induced by LPS in BECs in TNF- α -independent manner.

In conclusion, LPS, a gram-negative bacterial component, induced the overexpression of MUC2 and MUC5AC in cultured murine BECs via production of TNF- α and activation of PKC. This finding may explain how bacterial infection is involved in the altered and enhanced mucin secretion in the intrahepatic biliary tree and in turn relates to the lithogenesis of hepatolithiasis.

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