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Cathelicidin Stimulates Colonic Mucus Synthesis by Up-Regulating *MUC1* and *MUC2* Expression Through a Mitogen-Activated Protein Kinase Pathway

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Abstract Mucus forms the physical barrier along the gastrointestinal tract. It plays an important role to prevent mucosal damage and inflammation. Our animal study showed that antibacterial peptide 'cathelicidin' increased mucus thickness and prevented inflammation in the colon. In the current study, we examined the direct effect and mechanisms by which the peptide increased mucus synthesis in a human colonic cell line (HT-29). Human cathelicidin (LL-37) dose-dependently (10–40 μg/ml) and significantly stimulated mucus synthesis by increasing the D-[6-^{3}H] glucosamine incorporation in the cells. Real-time PCR data showed that addition of LL-37 induced more than 50% increase in *MUC1* and *MUC2* mRNA levels. Treatment with *MUC1* and *MUC2* siRNAs normalized the stimulatory action of LL-37 on mucus synthesis. LL-37 also activated the phosphorylation of mitogen-activated protein (MAP) kinase in the cells. A specific inhibitor of the MAP kinase pathway, U0126, completely blocked the increase of *MUC1* and *MUC2* expression as well as mucus synthesis by LL-37. Taken together, LL-37 can directly stimulate mucus synthesis through activation of *MUC1* and *MUC2* expression and MAP kinase pathway in human colonic cells. J. Cell. Biochem. 104: 251–258, 2008.

Key words: cathelicidin; mucus; mucin; mitogen-activated protein kinase

The gastrointestinal (GI) tract is covered by a viscous mucus layer. This layer works primarily as a lubricant ensuring low friction between luminal contents and the epithelium, and secondly it acts as a physical barrier protecting the underlying epithelium from mechanical, chemical, enzymatic, and microbial damages. It further protects the host from pathogenic infection by its store of co-secreted immunoglobulin A [Neutra and Forstner, 1987]. The gel-forming properties of mucus are provided by its building blocks, mucins. Mucins are macromolecuolar glycoproteins encoded by mucin (*MUC*) genes. Currently, 20 human *MUC* genes have been identified [Dekker et al., 2002]. Mucins are generally divided into two main groups: secreted mucins including *MUC2* and *MUC6* that are made and secreted by goblet cells; and membrane-bound mucins such as *MUC1*, *MUC3*, and *MUC4* that are anchored in the glycocalyx. They are heavily glycosylated and strongly charged as a consequence of sialylation and/or sulphation which are essential for the thickness and gel-like properties of mucus [Forstner et al., 1995].

Increasing evidence suggests that abnormal mucus secretion is the hallmark for the pathogenesis of several diseases. Mucus hypersecretion is one of the major causes of the inflammatory airway diseases like chronic bronchitis, asthma, and cystic fibrosis [Kim, 1997]. Marked alternations in the colonic mucus have also been reported in ulcerative colitis (UC) and colonic cancer [Corfield et al., 1996; Kim et al., 1996]. However, the mechanisms regulating these changes are still not understood. The importance of mucus alternation in

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the initiation or perpetuation of UC has been widely studied over decades. UC is one major form of inflammatory bowel disease (IBD) which is primarily characterized by superficial inflammation of the large intestine. While the cause of UC remains obscure, reduction of colonic mucus has been well documented and suggested to be one of the possible etiologies. During the active stage of UC, the mucus layer is severely disrupted and discontinuous with a 60–70% decrease in the thickness [Pullan et al., 1994]. This is linked to the depletion of goblet cells and down-regulation in mucin production which could be influenced by immunological or bacterial factors during inflammation.

In vitro study revealed that pro-inflammatory cytokines interleukin (IL)-1, IL-6, or tumor necrosis factor (TNF)-α resulted in an increased synthesis of *MUC2* and other secretory mucins whereas incubation of individual cytokine additionally resulted in decreased and altered glycosylation in human goblet cell-like cells [Enss et al., 2000]. The elevated number of bacteria detected within the colonic mucus layer of UC patients poses another threat to the intestinal mucosa. Partial degradation of the mucus by increased levels of mucinase [Dwarakanath et al., 1995], sulphatase [Tsai et al., 1995], and extracellular glycosidases of bacterial origin may raise the exposure of the epithelium to pathogens and other luminal substances and sustain the chronic character of UC [Corfield et al., 1992]. These findings reinforce the idea that enhancing the mucosal barrier could be an important therapeutic strategy therapy in treating IBD patients. Indeed, our recent study demonstrated that mouse cathelicidin (mCRAMP) given intrarectally in mice could attenuate dextran sulfate sodium (DSS)-induced colitis through the preservation of the mucus-secreting layer during inflammation [Tai et al., 2007]. This was accompanied by an up-regulation of the *MUC1*, *MUC2*, *MUC3*, and *MUC4* genes expression. However, it is interesting to know whether the peptide can directly stimulate mucus secretion through the expression of these mucin genes in colon cells and also the mechanism involved in such action. In this connection, it was reported that *MUC2*, the most predominant secretory mucin in the colon [Hayashi et al., 2001], could be stimulated through a mitogen-activated protein (MAP) kinase pathway [Iwashita et al., 2003]. It is therefore likely that cathelicidin could act through such pathway and increase mucus synthesis in the colon.

MATERIALS AND METHODS

Cell Culture

HT-29, a cell line derived from the human colorectal adenocarcinoma, was obtained from the American Type Culture Collection (Manassas, VA) and maintained at 37°C in RPMI-1640 (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum, 0.2 g/L streptomycin and 0.1 g/L penicillin in a 5% CO_2 incubator. Cells were collected before confluence following 5–6 days of subculture and subsequently used for experiments.

Exposure of Cells to LL-37

The full-length of mature human cathelicdin, LL-37, was purchased from Invitrogen and dissolved in the phosphate buffered saline (PBS) for treatment. U0126 (Sigma–Aldrich, St. Louis, MO) in dimethyl sulfoxide was used as the MAP kinase kinase inhibitor. The cells were seeded in either a 6-well plate at 1×10^4 cells/well or in a 24-well plate at 1×10^5 cells/well. Various concentrations (0, 10, 20, and 40 μg/ml) of LL-37 with or without U0126 were added after 16 h.

Determination of Mucus Synthesis in HT-29 Cells

The amount of mucus synthesis was determined by measuring incorporation of D-[6-^{3}H] glucosamine into mucosal glycoprotein according to a modified method of Ma et al. [2000]. In brief, cells in 24-well culture plates were washed with PBS, followed by incubation with 0.5 ml of the medium containing [H^3]-glucosamine HCl (Amersham, Little Chalfont, UK) in the presence of LL-37 for different time intervals. At the end of incubation, medium was aspirated and discarded. It was found that the amount of mucus glycoprotein secreted from cells into the medium was very low over the incubation period. The remaining cells were washed twice with PBS. The cells were then solubilized with 0.4 ml of 0.3 M NaOH and neutralized with 0.4 ml of 0.3 M HCl. The resulting aliquot was precipitated by the addition of 0.5 ml of 50% TCA. Pellets were washed twice with 10% TCA and once with chloroform-methanol solution (1:1, v:v). The dried pellets

were dissolved in 25 μl of DMSO and the fractions were subjected to Sepharose® CL-4B (Sigma Chemicals, St. Louis, MO) column chromatography for isolation of mucin, as previously described [Takahashi and Okabe, 1998]. The radioactivity in the void fractions was measured as the amount of mucus synthesized by cells. Mucus synthesis was expressed as [H^3]-glucosamine incorporation (cpm) per 1×10^5 cells.

Quantitative Analysis of Mucin Gene Expression

The HT-29 cells were cultured in a six-well plate and treated with LL-37 for 24 h. The total cellular RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. A total of 2.5 μg extracted RNA was used as the template for complementary DNA (cDNA) synthesis using the Thermoscript reverse transcription-polymerase chain reaction system (Invitrogen).

Quantitative real-time PCR was performed for *MUC1*, *MUC2*, *MUC3*, *MUC4*, and β-*actin* using the following primers pairs: *MUC1*, sense primer 5′-TGCTTACAGTTGTTACGGGTTC-3′ and antisense primer 5′-CCCCAGTAGACAAAGCATTC-3′; *MUC2*, sense primer 5′-GTCAGCACCCCGCACTAC-3′, and antisense primer 5′-AGCTCCAGCATGAGTGCAT-3′; *MUC3*, sense primer 5′-TGGATCTAGATGTAGTGGAGACC-3′ and antisense primer 5′-TGCAAAAATCTTCTGCATCTG-3′; *MUC4*, sense primer 5′-TTCAGTCCAACTGTCAACCTAGA-3′, and antisense primer 5′-CCACCGAGGCGTTGACTT-3′; β-*actin*, sense primer 5′-CTTCCTGGGCATGGAGTC-3′ and antisense primer 5′-GCAATGATCTTGATCTTCATTGTG-3′. The cDNA was amplified using iQ SYBR Green supermix (Bio-Rad Laboratories, Hercules, CA) on the iCycler thermal cycler (Bio-Rad), programmed for 95°C for 10 min, then 40 cycles of denaturation (95°C for 15 s), annealing (59°C for 15 s), and extension (72°C for 15 s). The amplification results were detected and analyzed using the iQ5 real-time PCR detection system (Bio-Rad). The gene signals were standardized against the corresponding β-*actin* signals and results were expressed as the ratio of each molecule to β-*actin*.

Conventional RT-PCR was carried out using conditions as follows: the template cDNA was first denatured at 94°C for 5 min. During 40 cycles of amplification, the denaturation step was at 94°C for 1 min, the annealing step at 55°C for 1 min, and the extension step at 72°C for 1 min. The final extension step was at 72°C for 7 min. The PCR products were electrophoresed on 1.5% (w/v) agarose gels containing 0.5 g/ml ethidium bromide. Gel photographs were then analyzed semiquantitatively in a multianalyzer (Bio-Rad).

Knockdown of *MUC1* and *MUC2* by small interfering RNA

Small interfering RNA (siRNA) targeting *MUC1* and *MUC2* (*MUC1*-siRNA and *MUC2*-siRNA) was obtained from Santa Cruz (CA). Transfection was performed using Oligofectamin reagent (Invitrogen) according to the manufacturer's instructions. The efficacy of *MUC1* and *MUC2* knockdown was assessed by convectional RT-PCR. Assays were performed 2 days after transfection.

Western blot

Cells seeded in a six-well plate were treated with 40 μg/ml of LL-37 for various time periods (0, 5, 10, 15, 30, and 60 min). Total cell lysates were prepared and equivalent amounts of protein were analyzed by Western blot as described previously [Yang et al., 2006]. Phospho-p44/42 MAP kinase (Thr202/Tyr204) polyclonal antibody and p44/42 MAP kinase polyclonal antibodies from Cell Signaling Technology (MA) were used to detect the activated and basal forms of MAP kinase.

Statistical Analysis

All data were expressed as means ± standard error (SE). Means were compared by the use of ANOVA and a P-value of < 0.05 was considered to be statistically significant.

RESULTS

Human Cathlicidin (LL-37) Increased Mucus Synthesis

Previous study showed that mouse cathelicidin prevented experimental colitis and increased mucus level in the colon [Tai et al., 2007]. The present study demonstrated the direct effect of a human cathelicidin, LL-37, on mucus secretion from colonic cells. LL-37 incubation at the doses from 10 to 40 μg/ml for 12, 24, or 48 h, dose- and time-dependently increased mucus synthesis. Significant effect was observed at the 24 and 48 h time points

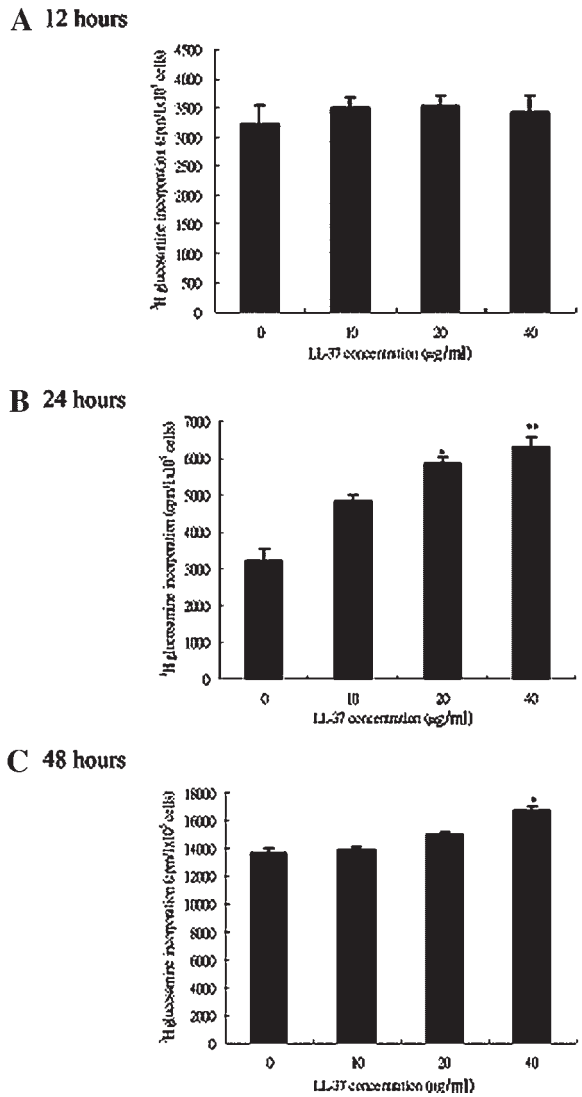

Fig. 1. Effects of LL-37 on mucus synthesis in HT-29 cells. Cells were incubated with various concentrations of LL-37 for 12 h (**A**), 24 h (**B**), and 48 h (**C**). Mucus synthesis was determined by the amount of [^{3}H]-glucosamine incorporation per 1×10^5 cells. Values are mean ± SE of 12 samples. $^*P < 0.05$ and $^{**}P < 0.05$ when compared with the control group.

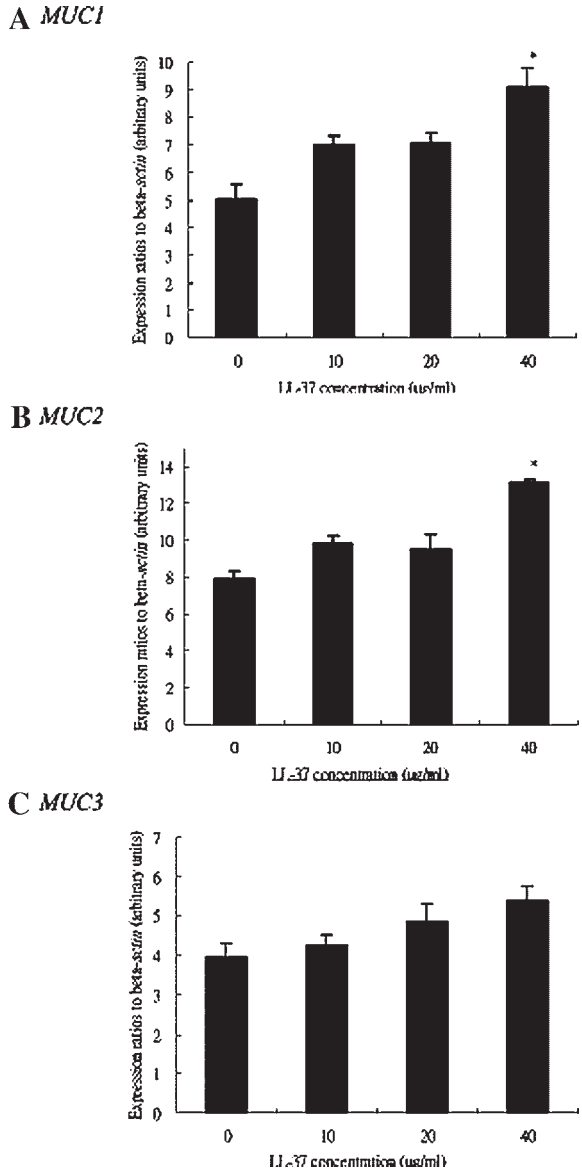

Fig. 2. Effects of LL-37 on the expression of mucin genes in HT-29 cells, (**A**) *MUC1*, (**B**) *MUC2*, and *MUC3*. The cells were cultured with 0, 10, 20, and 40 μg/ml of LL-37 for 24 h. RNA was extracted at the end of treatment and the gene expression of each target gene was determined by real-time PCR which was standardized against the expression of β-*actin*. Values are mean ± SE of 12 samples. $^*P < 0.05$ when compared with the control group.

(Fig. 1). The stimulatory action peaked at 24 h (Fig. 1B) and declined thereafter (Fig. 1C).

Up-Regulation of *MUC1* and *MUC2* Expression by LL-37

To clarify the action of LL-37 on mucin gene expression, we monitored mucin gene expression by determining the mRNA levels of *MUC1*, *MUC2*, *MUC3*, and *MUC4* in the cells. LL-37 treatment at the highest dose, 40 μg/ml, significantly increased the expression of *MUC1* and *MUC2*, by about 80% and 60%, respectively, after 24 h of incubation (Fig. 2A,B). The peptide tended to dose-dependently increase *MUC3* expression but no significant effect was observed. There was no *MUC4* gene expression in the HT-29 cells (data not shown).

LL-37 Stimulated Mucus Synthesis Via Up-Regulating *MUC1* and *MUC2* Expression

To confirm the stimulatory action of LL-37 on mucus synthesis was mediated via the increased expression of both *MUC1* and *MUC2*, siRNAs

for both genes were employed to knock down the mucin gene expression and determined the mucus synthesis after LL-37 incubation. The introduction of *MUC1* or *MUC2* siRNA could reduce the respective mRNA level by about 64% (Fig. 3A,B). Individual siRNA partially reversed the stimulatory action of LL-37 on mucus synthesis in HT-29 cells (Fig. 3C). In addition, down-regulation of both *MUC1* and *MUC2* genes (50% and 53%, respectively) together by treatment with both siRNAs (Fig. 4A) could further lessen the stimulatory action of LL-37 on mucus synthesis back to the basal level (Fig. 4B), indicating that both mucin genes are wholly involved in the stimulatory action of LL-37 on colonic mucus synthesis.

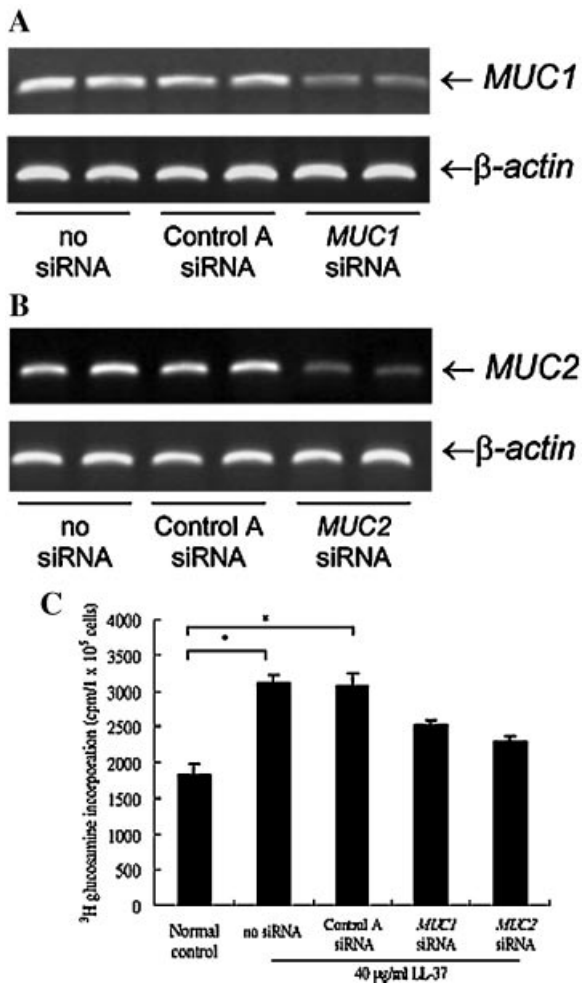

Fig. 3. Influences of down regulation of specific mucin genes by siRNAs on LL-37-induced mucus synthesis in HT-29 cells. Effects of knockdown of endogenous mucins by specific siRNAs, (**A**) *MUC1* and (**B**) *MUC2*. HT-29 cells were treated with siRNAs targeting *MUC1* and *MUC2*, respectively. The levels of mucin transcript were detected by conventional RT-PCR. **C**: Effects of knockdown of mucin genes on LL-37-induced mucus synthesis. HT-29 cells were treated with or without siRNAs targeting particular mucin genes for 48 h and then incubated with or without 40 μg/ml of LL-37 for another 24 h. Mucus synthesis was determined by [^{3}H]-glucosamine incorporation as before. Values are mean ± SE of 12 samples. *$P < 0.05$ when compared with the normal control group.

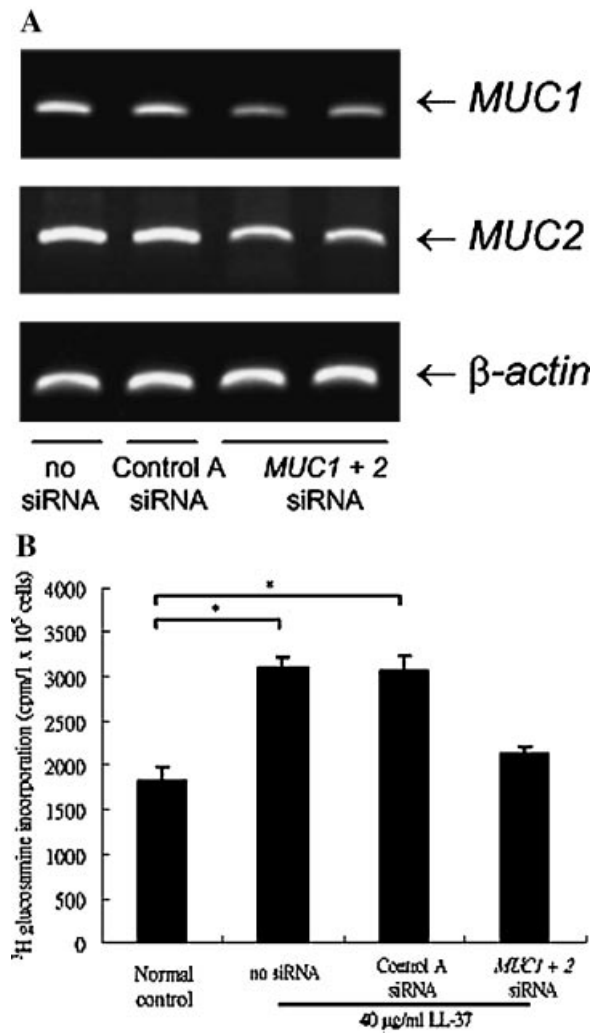

Fig. 4. Influences of down regulation of mucin genes by siRNAs on LL-37-induced mucus synthesis in HT-29 cells. **A**: Effects of knockdown of endogenous mucins by treatment of both *MUC1* and *MUC2* siRNAs. HT-29 cells were treated with siRNAs targeting *MUC1* and *MUC2*. The levels of mucin transcript were detected by conventional RT-PCR. **B**: Effects of knockdown of both mucin genes on LL-37-induced mucus synthesis. HT-29 cells were treated with or without siRNAs targeting both mucin genes for 48 h and then incubated with or without 40 μg/ml of LL-37 for another 24 h. Mucus synthesis was determined by [^{3}H]-glucosamine incorporation as before. Values are mean ± SE of 12 samples. *$P < 0.05$ when compared with the normal control group.

LL-37 Activated Phosphorylation of MAP Kinase in HT-29 Cells

A previous study showed that MAP kinase pathway is involved in the induction of *MUC2* mRNA expression by cytokines [Iwashita et al., 2003]. This finding prompted us to examine the causal relationship between activation of MAP kinase and mucus synthesis by LL-37. Interestingly, LL-37 activated the phosphorylation of MAP kinase as early as 10 min after incubation and peaked at 15 min, whereas the level of total MAP kinase was not affected (Fig. 5).

Phosphorylation of MAP Kinase Involved in LL-37-Mediated Mucin Gene Expression and Mucus Synthesis

To examine further whether phosphorylation of MAP kinase is required for the stimulatory action of LL-37 on mucus synthesis, a MAP kinase kinase inhibitor, U0126, which can inhibit the phosphorylation of MAP kinase, was used. Incubation of the inhibitor alone did not alter mucus synthesis. However, U0126 pretreatment could completely abolish the stimulatory effect of LL-37 on mucus synthesis (Fig. 6). We also studied whether phosphorylation of MAP kinase is responsible for the stimulatory action of LL-37 on mucin gene expression. Results showed that addition of U0126 totally reversed the stimulatory effects of LL-37 on *MUC1* and *MUC2* expression (Fig. 7).

DISCUSSION

Cathelicidin, an antimicrobial peptide of the innate immune systems, regulates microbial growth, wound healing, and inflammation [Tai

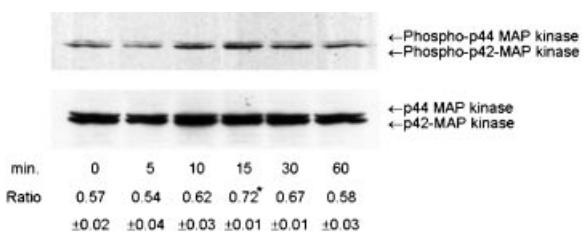

Fig. 5. Activation of mitogen-activated protein (MAP) kinase by LL-37 in HT-29 cells. The cells were incubated with 40 μg/ml of LL-37 for the time indicated. Activation of MAP kinase was detected by Western blot using an antiphospho MAP kinase antibody. Total MAP kinase was also detected as a loading control. The respective calculated phospho-MAP kinase to total MAP kinase ratios were shown below the time point. $*P < 0.05$ when compared with the ratio before LL-37 addition ($n = 3$).

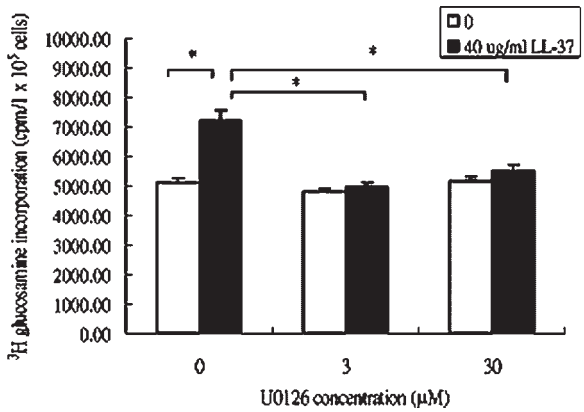

Fig. 6. Effects of a MAP kinase kinase inhibitor on mucus synthesis in HT-29 cells. The cells were incubated with LL-37 (40 μg/ml) with or without the MAP kinase kinase inhibitor, U0126 (3 and 30 μM), for 24 h. Mucus synthesis was determined by [^{3}H]-glucosamine incorporation assay as before. Values are mean ± SE of six samples. $*P < 0.05$ when compared with the respective group.

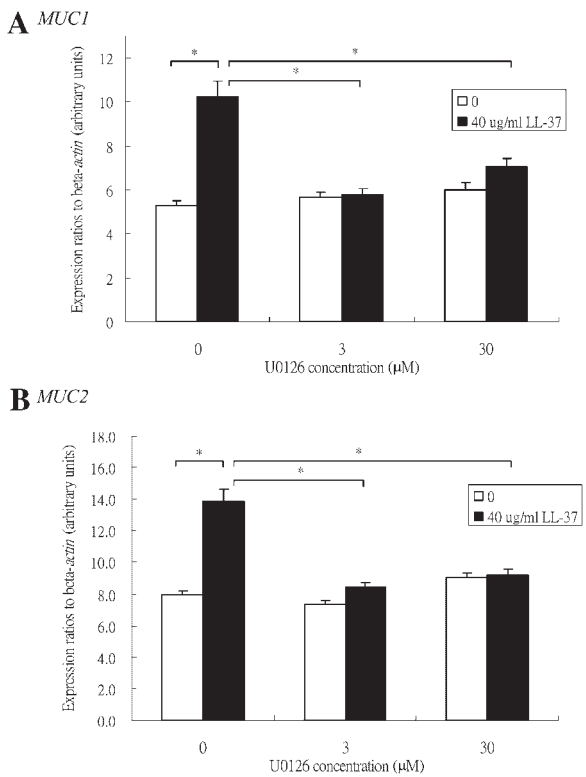

Fig. 7. Effects of a MAP kinase kinase inhibitor on gene expression in HT-29 cells, (**A**) *MUC1* and (**B**) *MUC2*. HT-29 cells were cultured with LL-37 (40 μg/ml) with or without the MAP kinase kinase inhibitor, U0126 (3 and 30 μM). RNA was extracted after 24 h and the gene expression was detected using real-time PCR as before. Values are mean ± SE of six samples. $*P < 0.05$ when compared with the indicated group.

et al., 2006]. Other report reveals that this peptide can alter inflammation by neutralizing the toxic effects of lipopolysaccharide [Larrick et al., 1995]. Our recent study further demonstrated that cathelicidin ameliorated DSS-induced experimental colitis in murine animals [Tai et al., 2007]. In this study, one of the pronounced actions of cathelicidin was its stimulatory effect on colonic mucus thickness and mRNA expression of different mucin genes (*MUC1*, *MUC2*, *MUC3*, and *MUC4*). However, it is still unknown whether cathelicidin can directly stimulate mucus synthesis through the activation of mucin gene expression, if any, by a potential signal transduction in human colonic epithelial cells.

In the current report, we demonstrated for the first time that LL-37 dose-dependently stimulated mucus production after 24 h of treatment (Fig. 1B). Addition of 40 μg/ml of LL-37 markedly increased mucus synthesis by about 63% and also up-regulated *MUC1* and *MUC2* mRNA levels in HT-29 cells (Fig. 2). No alternation in *MUC3* and *MUC4* expression was detected. These findings indicate that both *MUC1* and *MUC2* genes could be involved in the stimulatory action of LL-37 on mucus synthesis in human colon cells. Similar findings were observed in vivo in which the action of mouse cathelicidin on mucus synthesis correlates with the increased expression of some mucin genes including *MUC1*, *MUC2*, *MUC3*, and *MUC4* in the mouse colonic mucosa [Tai et al., 2007]. The difference in *MUC3* and *MUC4* responses in the current and the previous studies [Tai et al., 2007] could be anticipated, as there are obvious differences in cell types and experimental conditions in these experiments.

To confirm whether up-regulation of these mucin genes is the major mechanism for LL-37 to stimulate mucus synthesis, experiments to modulate the expression of *MUC1* or *MUC2* in colon cells were performed. Knockdown of either *MUC1* or *MUC2* gene could partially abrogate the stimulatory effect of LL-37 on mucus synthesis. There was an additive effect when both genes were downregulated, in reversing the effect of LL-37 on mucus synthesis in the cells (Fig. 4B). Both *MUC1* and *MUC2* seem to play a concurrent role in the induction of mucus production.

The mechanism of how cathelicidin promotes mucin gene expression is unclear. It has been reported that inhibition of MAP kinase kinase reduces *MUC4* expression in rat adenocarcinoma cells [Zhu et al., 2000] and *MUC5AC* synthesis is stimulated through activation of MAP kinase in human pulmonary carcinoma cells [Takeyama et al., 2000]. Iwashita et al. [2003] further showed that *MUC2* could be stimulated by several cytokines via the MAP kinase pathway in human colonic cancer cells. Therefore, we hypothesize that LL-37 modulate *MUC1* and *MUC2* expression and eventually mucus synthesis through the phosphorylation of MAP kinase (Fig. 8). Indeed, LL-37 could activate MAP kinase phosphorylation (Fig. 5) and more importantly, addition of a specific inhibitor of MAP kinase kinase, U0126, effectively blocked the stimulatory actions of LL-37 on mucin gene expression as well as mucus synthesis (Figs. 6 and 7). It is likely that activation of MAP kinase is a crucial factor to stimulate mucus synthesis and protect against mucosal damage in the colon. Indeed, *MUC2*, the predominant secretory mucin in the colon, has been reported to be involved in colonic protection [Hayashi et al., 2001]. *MUC2* deficiency led to abnormal morphology, ulceration of epithelial cells, and a mild increase of inflammatory cells in the mouse colon [Van der Sluis et al., 2006]. Also, decrease in its expression was linked to the reduction of mucus layer in UC patients [Tytgat et al., 1996]. Most notably, *MUC2*-deficient mice frequently developed adenomas in the small intestine and colon which might progress to invasive adenocarcinomas [Velcich et al., 2002]. Further evidence showed that repeated cycle of injury and repair during inflammation in intestinal mucosa

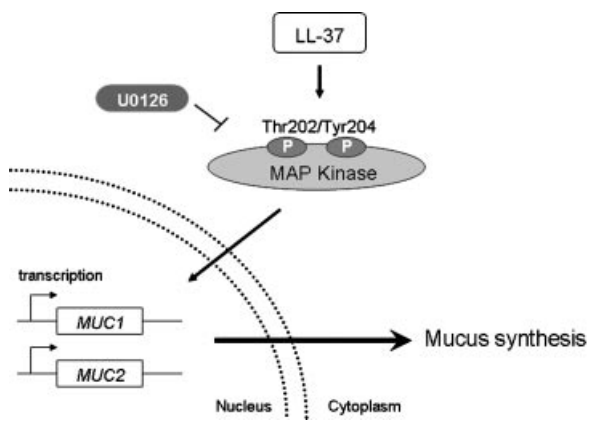

Fig. 8. Proposed mechanism for the mucus stimulation of LL-37 in HT-29 cells. Activation of MAP kinase results in up-regulated transcription of *MUC1* and *MUC2* which leads to stimulated mucus synthesis.

increases the risk of colorectal cancer [Liu et al., 2003]. In this regard, cathelicidin may be useful as a prophylactic therapy for colorectal cancer through blocking this repeated inflammatory cycle [Tai et al., 2007] and enhancing mucosal defensive barrier through mucus synthesis in the colon.

In conclusion, the present study provides a direct evidence to show the causal relationship between MAP kinase activity and mucin gene expression in the stimulatory action of cathelicidin on mucus synthesis. The peptide can stimulate mucus production by boosting the *MUC1* and *MUC2* mRNA expression through activation of MAP kinase phosphorylation. These findings demonstrate a substantial relevance in both basic and clinical applications for cathelicidin in treating IBD and perhaps also colorectal cancer.

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