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Regulation of IL-1β-Mediated MUC2 Gene in NCI-H292 Human Airway Epithelial Cells

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This study demonstrates for the first time the effects of IL-1β on the regulation of protein production as well as MUC2 gene transcription in cultured human airway epithelial cells. The effect of IL-1β on the regulation of MUC2 protein was determined by flow cytometric analysis. The expression level of MUC2 induced by IL-1β increased in a dose-dependent manner. MUC2 transcripts were detected after 2 h of exposure to IL-1β and reached maximal level after 8 h. Actinomycin D experiments indicated that the IL-1β-mediated MUC2 expression was controlled by transcriptional regulation. Both RT-PCR and FACS analysis showed that budesonide concomitantly attenuated IL-1β mediated MUC2 gene as well as protein production levels. Use of the glucocorticoid receptor antagonist, RU-486, restored the inhibitory effect of budesonide on the IL-1β-mediated MUC2 protein as well as gene. The data suggest that IL-1β up-regulates MUC2 gene by transcriptional regulation and that budesonide suppresses the IL-1β-medicated MUC2 expression via decreased transcriptional activation.

The normal human airway is coated with mucus glycoproteins. The respiratory mucus glycoprotein or mucin is an important component of the airway secretions. Airway inflammation is generally associated with mucus hypersecretion. Cytokines and other inflammatory mediators stimulate airway mucin hypersecretion, either directly or indirectly. Mucin is a high-molecular-mass glycoprotein which consist of as much as 80% carbohydrate side chains which are linked via the activities of glycosyltransferases to serine and threonine residues of the peptide backbone (1). Nine human mucin genes have been identified in various tissues such as respiratory, gastrointestinal, and reproductive tracts (1). MUC2, MUC5AC and MUC5B of nine mucin genes have been shown to be subject to regulation by a variety of inflammatory mediator, among them bacterial lipopolysaccharide (LPS) in airway epithelial cells (1). Many cytokines and other inflammatory mediators are important regulator in inflammatory airway diseases. Particularly, interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) act primarily as proinflammatory cytokines. Several studies of the effects TNF-α and LPS on mucin gene expression have been reported. They indicated that TNF-α and/or LPS up-regulate MUC2 and MUC5AC gene expression in NCI-H292 cells by transcriptional regulation (2–4). Recently Yoon *et al.* have been reported that TNF-α and IL-1β increase MUC8 mRNA levels with synergism, but they showed the combination of these cytokines did not change dramatically MUC2 gene in the normal human nasal epithelial cells (5).

IL-1β is one of most important multifunctional proinflammatory cytokines with an active role in acute and chronic airway inflammation (6, 7). IL-1β may also be involved in the pathogenesis of inflammatory airway disorders, such as asthma and bronchitis. To date, however, it remains unclear whether IL-1β affects mucin gene expression or mucus production in airway epithelial cells. Although several effective drugs have been developed that control airway mucus production, the glucocorticoids seem to be most effective. However, there are still very few reports about the effects of glucocorticoids on airway mucin and mucin genes expression (8).

We concentrated in our studies on the MUC2 gene for the following reasons. The MUC2 gene is only weakly expressed in normal respiratory epithelium, but it seems to be markedly up-regulated during airway inflammatory disease such as cystic fibrosis (3). Furthermore, several investigators have reported that cytokines, inflammatory mediators (3, 9–11), as well as bacterial LPS (3) regulated MUC2 gene.

The present study was undertaken to assess the effect of IL-1β on the regulation of the MUC2 gene and

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protein expression and to determine the effect of budesonide on MUC2 gene regulation by IL-1β in human airway epithelial cells. We developed a method to detect mucin protein by flow cytometry, since the mucin glycoprotein is a large and complex molecule that has been difficult to characterize either by biochemical or molecular method. Our findings demonstrate that IL-1β induces MUC2 gene expression and protein production in a dose- and time-dependent manner mainly by transcriptional regulation and that budesonide can down-regulate the MUC2 gene induction and mucin protein production by IL-1β.

EXPERIMENTAL PROCEDURES

Cell culture and experimental design. A human pulmonary mucoepidermoid carcinoma cell line, NCI-H292 (American Type Culture Collection, Rockville, MD) was seeded at a density of 1×10^6 cells into 6 well plates. Cultures were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C in a humidified atmosphere of 95% air and 5% CO_2. When cultures were confluent, the cells were incubated with RPMI 1640 medium containing 0.5% fetal calf serum for 24 h, after which they were rinsed with phosphate buffered saline (PBS) and exposed to the indicated concentrations of reagents subsequent human recombinant IL-1β (R&D Systems, Minneapolis, MN) treatment. Some cultures were pretreated with budesonide (Sigma, St. Louis, MO) for 1 h before exposed to IL-1β. After treatment of other cells for the indicated periods of time, total cellular RNA was extracted using a Tri-Reagent (Molecular Research Center, Cincinnati, OH). Some cultures were treated with 1 μg/ml actinomycin D and 20 μg/ml cycloheximide with or without each of the tested reagents to determine the requirements for gene expression at the transcriptional level or the *de novo* protein synthesis level, respectively. Control cultures remained untreated. IL-1β was dissolved with PBS containing 0.1% bovine serum albumin, and budesonide was dissolved in ethanol. The final concentrations of ethanol or other vehicle solvents in the medium were less than 0.1%.

RT-PCR analysis of the MUC2 gene. The method used to detect and quantify MUC2 mRNA level employed a modified technique of RT-PCR and has previously been described in detail (12). Briefly, total RNA was reverse transcribed into cDNA using random hexanucleotide primers and MULV reverse transcriptase (Perkin–Elmer, Morrisville, NC). The oligonucleotide primers for the PCR part of the procedure were designed based on the published sequences of human MUC2 (GenBank Accession No. L21998, 5′ primer: TGC CTG GCC CTG TCT TTG; 3′ primer: CAG CTC CAG CAT GAG TGC). The PCR parameters were 35 cycles of denaturation (95°C, 1 min), annealing (60°C, 1 min) and extension (72°C, 1 min) in the presence of 2.5 mM $MgCl_2$, concluding with a 20 min extension at 72°C. The MUC2 DNA fragment generated was the expected a 440 bp in size. The oligonucleotide primers for the β2 microglobulin (β2M, used as a control gene for the RT-PCR) were purchased from Clontech (Palo Alto, CA) and generated a 335 bp PCR fragment. Specific amplification of MUC2 was confirmed by sequencing (dsDNA Cycle Sequencing System, Gibco BRL, Grand Island, NY). PCR products were separated by electrophoresis through a 2% agarose gel in 1% TBE buffer containing 50 ng/ml of ethidium bromide and photographed using a Polaroid type 55 film. The intensity of the bands was analyzed with a densitometer.

MUC2 protein analysis by FACS. Indirect immunofluorescence assays were performed by incubating the cells with MUC2 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and, subsequently, with FITC-conjugated rabbit anti-mouse immunoglobulin (Santa Cruz Biotechnology). The cells were fixed with 0.1% paraformaldehyde and analyzed using a FACS Calibur (Becton Dickinson, Mountain View, CA).

RESULTS

Induction of the MUC2 mucin gene by IL-1β. In order to define the effect of IL-1β on steady-state MUC2 mRNA levels, RT-PCR experiments were performed on the NCI-H292 cells. Confluent cultures of NCI-H292 cells were incubated with IL-1β in the range of 0.02–20 ng/ml at 37°C for 8 h. Total RNA was then extracted and subjected to RT-PCR analysis. As the dose of IL-1β was increased from 0.02 to 20 ng/ml, there was a parallel increase in the MUC2 mRNA level. RT-PCR products of β2M mRNA are used as internal controls. As shown in Fig. 1A, 2 and 20 ng/ml of IL-1β induced mRNA level of MUC2 approximately 3-fold as compared to the control treated with vehicle alone.

To determine whether IL-1β induced mRNA level of MUC2 in a time dependent, we examined the mRNA levels of MUC2 after various lengths of exposure to IL-1β (Fig. 2). MUC2 transcript levels started increasing after 2 h of exposure to IL-1β and continued to increase for up to 24 h. The maximum level of MUC2 mRNA was reached after 8 h.

Production of mucin protein stimulated by IL-1β. To confirm the regulation of mucin production by IL-1β at the protein level, we used FACS analysis with anti-MUC2 antibody. Western blotting using anti-mouse MUC2 antibody did not produced detectable results. In the flow cytometric analysis, untreated cells appeared like a normal population displaying various degrees of mucin protein production. However, treatment with IL-1β shifted the mucin protein immunofluorescence of the cells to a higher intensity (Fig. 1B). Thus, we could conclude that treatment of the cells with IL-1β increased mucin protein expression on the cell surface. This up-regulation is reflected in the shifts seen corresponding FACS histogram (Fig. 1B). The results, therefore, indicated that the MUC2 mRNA accumulation pattern caused by IL-1β is correlated with FACS data of the mucin protein production.

Effect of inhibitors of transcription and translation on the IL-1β-induced MUC2. In order to determine whether the IL-1β regulated MUC2 expression involved transcriptional and/or translational mechanism, we treated the NCI-H292 cells with IL-1β after the pretreatment of the cells with actinomycin D (1 μg/ml) or cycloheximide (20 μg/ml) for 1 h. Actinomycin D, an inhibitor of RNA synthesis, suppressed MUC2 mRNA transcription induced by IL-1β, while cycloheximide did not inhibit MUC2 mRNA accumulation (Fig. 3). These results suggest that the IL-1β mediates MUC2 up-regulation via transcriptional activation and that this dose not require *de novo* protein synthesis.

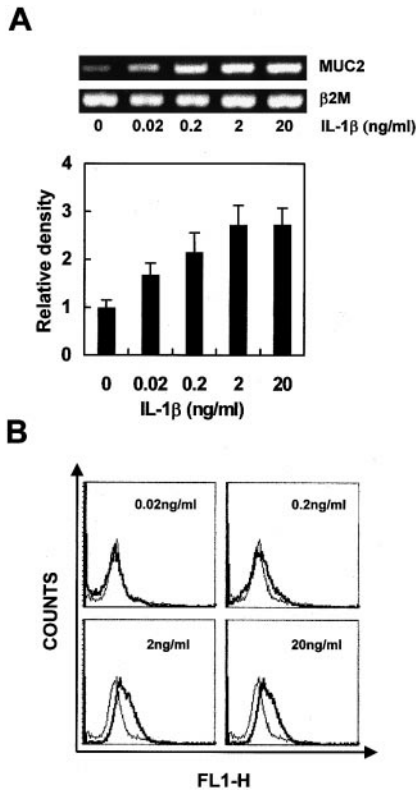

FIG. 1. Effect of IL-1β on induction of MUC 2 gene in cultured NCI-H292 cells. (A) NCI-H292 cells were treated with various concentrations of IL-1β. The mRNA levels of MUC2 and β2M were determined by RT-PCR. The size of PCR product of MUC2 is 440 bp. The positive control gene, β2M was not affected by IL-1β. The amounts of the RT-PCR products of MUC2 were quantified using scanning densitometry and expressed relative to the densities of β2M by the ratio of MUC2/β2M DNA bands. The data represent average values three independent experiments plus standard deviation. (B) FACS analysis of MUC2 mucin production in NCI-H292 cells treated with various concentrations of IL-1β. Immunofluorescence staining was performed for control and IL-1β-treated NCI-H292 cells. Each panel shows the fluorescence intensities on a log-scale (*X* axis) versus the number of cells (*Y* axis). Thin line histograms represent control and thick line histograms represent mucin protein fluorescence. The results were confirmed with three independent experiments.

Effect of budesonide on MUC2 induction by IL-1β. Pharmacological approaches to controlling excessive mucus production found a few useful drugs; among them, budesonide is one of more effective compounds. In order to determine whether budesonide affected MUC2 mRNA induction by IL-1β, we evaluated the MUC2 mRNA level in NCI-H292 cells pretreated with budesonide before addition of IL-1β. The MUC2 mRNA level induced by IL-1β was down-regulated in a manner dependent on the budesonide dose (Fig. 4). In addition, glucocorticoid receptor antagonist (RU 486) reversed the inhibitory effect of budesonide on MUC2 mRNA induced by IL-1β (Fig. 5A). To confirm suppression of budesonide-mediated mucin protein production, we carried out into FACS analysis (Fig. 5B). These

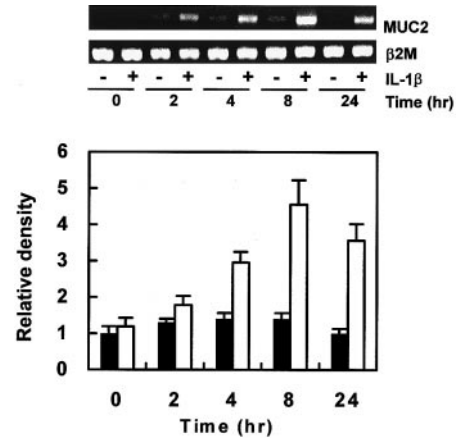

FIG. 2. Time course of MUC2 gene induction by IL-1β. NCI-H292 cells were treated with 20 ng/ml IL-1β for the indicated times (0, 2, 4, 8, and 24 h). Control cultures were sham-treated. The steady-state mRNA levels of MUC2 and β2M were determined by RT-PCR using total RNA. Relative densities are expressed as the ratio of MUC2/β2M DNA. The data represent average of three independent experiments plus standard deviation.

results of mucin protein production were consistent with the RT-PCR data for the MUC2 mRNA.

DISCUSSION

In this study, we demonstrated that IL-1β mediated MUC2 up-regulation was dose- and time-dependent and that its regulation was controlled mainly by transcriptional regulation in NCI-H292 cells which have been widely used as a model cells in which to investigate mucin gene regulation (2–4, 9). Exposure of NCI-H292 cells to 2 and 20 ng/ml IL-1β for 8 h increased MUC2 mRNA about 3-fold in comparison to control.

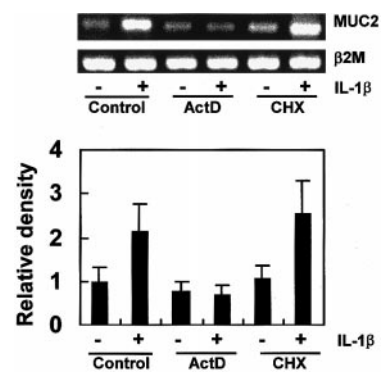

FIG. 3. Effect of transcription and translation inhibitors on the IL-1β induction of the MUC2 gene. NCI-H292 cells were just treated with IL-1β (20 ng/ml) or pretreated with actinomycin D (1 μg/ml) and/or cycloheximide (20 μg/ml) for 1 h before addition of IL-1β. Relative densities of the bands were calculated and converted to ratios of MUC2/β2M. The data represent average value of three independent experiments plus standard deviation.

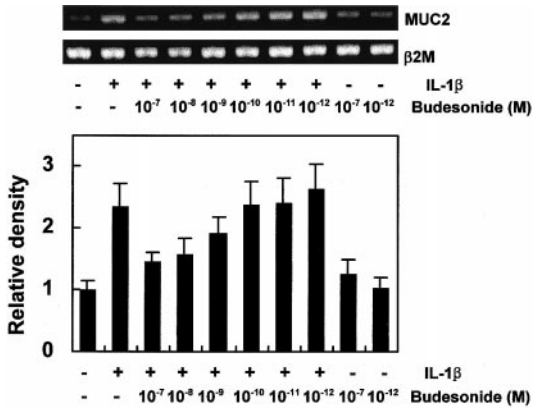

FIG. 4. Effect of budesonide on IL-1β induced MUC2 gene expression. NCI-H292 cells were cotreated with IL-1β (20 ng/ml) and various concentrations of budesonide for 8 h. Relative densities of the bands were determined and converted to ratios of MUC2/β2M. The data represent average value of three independent experiments plus standard deviation.

The MUC2 mRNA began to increase after 2 h, peaked at 8 h, and persisted for 24 h.

The production level of mucin protein is difficult to determine, because mucin is such a high-molecular mass glycoprotein. To date, mucin protein production has been determined via immunoblot, immunohistochemical stain and by FACS. Immunoblot and immunohistochemical staining analysis were not useful to detect MUC2 mucin protein using commercial MUC2 antibody. FACS analysis offers several advantages over the other methods used to detect and quantify specific mucin protein production. Therefore, we first established specific expression of mucin protein by FACS, which we subsequently found to be consistent with RT-PCR results.

There have been a few reports suggesting that cytokines and inflammatory mediators regulate the expression of mucin genes. Increases in MUC2 and/or MUC5AC mRNA accumulation have been observed in several instances such as: upon TNF-α treatment of NCI-H292 cells (9), *Pseudomonas aeruginosa* derived LPS treatment of HM3 and NCI-H292 cells (2–4), neutrophil elastase treatment of A549 cells (13), and acrolein treatment (14). However, there have been no reports yet about the effect of IL-1β on MUC gene transcription and mucin secretion. We focused on the regulation of the MUC2 gene expression and mucin secretion induced by IL-1β in cultured airway epithelial cells.

Glucocorticoids are effective inhibitors of IL-1 transcription. They are also effective drugs used to reduce mucin hypersecretion of airway inflammatory disease such as asthma and bronchitis. Glucocorticoids regulate transcription of responsive genes though the actions of glucocorticoid receptors (GR) in the cytoplasm (15). Upon binding of steroid molecules, the steroid/GR complex translocates to the nucleus where it binds to the glucocorticoid response element (GRE). Glucocorticoids can also affect mRNA stability modulating steady-state levels of specific mRNAs posttranscriptionally (16). We found that budesonide suppressed the IL-1β mediated mucin protein production as well as MUC2 gene induction. A glucocorticoid receptor antagonist (RU 486) blocked this inhibitory effect of budesonide on MUC2 gene transcription. Our findings were thus similar to the result that dexamethasone suppressed mucus production, MUC2 and MUC5AC gene expression in NCI-H292 cells reported by Kai *et al.* (8). On the other hand, these results stand in contrast to a report of Gollub *et al.* who showed that dexamethasone up-regulated MUC4 and MUC5AC in an Ishikawa epithelial cell line which was derived from an endome-

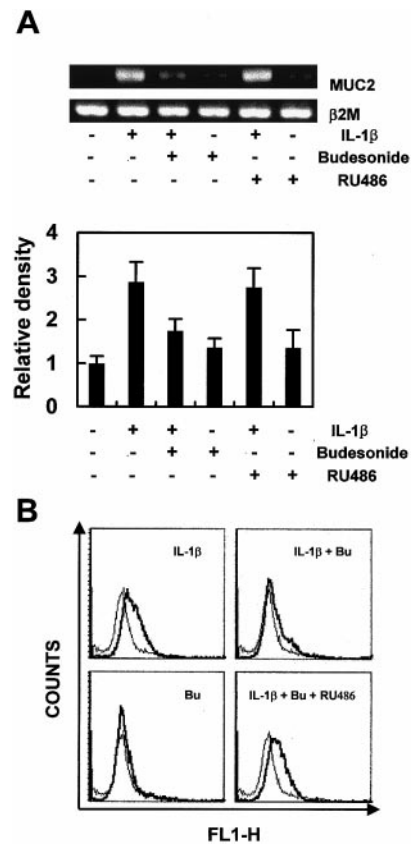

FIG. 5. Recovery effect of RU-486 on the MUC2 gene and protein suppression by budesonide. (A) NCI-H292 cells were treated with IL-1β (20 ng/ml) alone, with budesonide (10^{-8} M) alone, or after pretreatment with a combination of budesonide and RU 486 (10^{-7} M) for 1 h before addition of IL-1β. Relative densities of bands were determined and the ratio of MUC2/β2M DNA calculated. The data represent average three independent experiments plus standard deviation. (B) FACS analysis of mucin protein production in NCI-H292 cells treated with RU 486 and/or budesonide in combination with IL-1β. Immunofluorescence staining of control and IL-1β treated NCI-H292 cells are shown. Each panel shows fluorescence intensity (*X* axis) versus cell number (*Y* axis). Thin line histograms represents control fluorescence and thick line histograms mucin protein fluorescence. The results were confirmed three independent experiments.

trial adenocarcinoma (17). The discrepancy between the two observations of glucocorticoid effects may possibly be explained by differences of regulation of mucin gene according to the cell, glucocorticoid, and mucin gene types.

In conclusion, we report here that IL-1β up-regulates MUC2 gene expression and mucin protein production in a concentration- and time-dependent manner and that budesonide inhibits MUC2 gene and mucin protein induced by IL-1β in NCI-H292 cells. Further studies of the mucin gene expression will be needed to learn the details of the mechanisms by which the mucin gene expression is stimulated by IL-1β and inhibited by glucocorticoids in a variety of epithelial cell lines including the normal airway epithelium.

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