Clarithromycin inhibits overproduction of muc5ac core protein in murine model of diffuse panbronchiolitis

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Am J Physiol Lung Cell Mol Physiol 285: L847–L853, 2003. First published June 20, 2003; 10.1152/ajplung.00216.2002.—Long-term treatment of macrolide antibiotics is considered an effective treatment for diffuse panbronchiolitis (DPB). Although hypersecretion is a common feature of this disease, and it is known that macrohides inhibit mucin production, the mechanism of the effect on mucin production is unclear. The aim of our study was to determine the production of muc5ac core protein, a major core protein of mucin in airway secretion, and the effect of clarithromycin treatment on such production in a mouse model mimicking DPB. Alcian blue-periodic acid-Schiff-positive cells were detected in the lungs of Pseudomonas aeruginosa-infected mice. Western blots of these mice showed muc5ac glycoprotein at day 1 and increased progressively from day 4 to day 14 after inoculation of bacteria. Clarithromycin (10 mg·kg⁻¹·day⁻¹ for 7 days) significantly reduced the muc5ac expression at both the mRNA and protein levels. To investigate the role of molecules upstream in muc5ac regulation, we examined the role of mitogen-activated protein kinase. Extracellular signal-regulated kinase 1/2 phosphorylation increased in the infected lung and decreased after treatment. Our results suggest that overproduction of muc5ac plays an important role in the pathogenesis of DPB and that clinical improvement following macrolide therapy seems to involve, at least in part, its inhibition of mucin overproduction, through modulation of intracellular signal transduction.

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have also established a new murine model of chronic *Pseudomonas aeruginosa* respiratory tract infection mimicking DPB (41). As an extension to these studies, we used our murine model in the present study to analyze the extent of muc5ac production in the airways and effect of macrolide treatment on such secretion in mice with DPB-like respiratory infection.

**MATERIALS AND METHODS**

**Animal model of DPB.** Six-week-old, specific pathogen-free, male ddY mice were purchased from Shizuoka Agricultural Cooperative Association Laboratory Animals (Shizuoka, Japan). All animals were housed in a pathogen-free environment and received sterile food and water in the Laboratory Animal Center for Biomedical Science at Nagasaki University. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of our institution. Mice were infected with mucoid *P. aeruginosa* NUS10, a clinical isolate from sputum of patients at Nagasaki University Hospital. The bacteria were stored at −80°C in brain-heart infusion broth (BBL Microbiology Systems, Cockeysville, MD) supplemented with 10% (vol/vol) glycerol and 5% (wt/vol) skim milk (Yukijirushi, Tokyo, Japan) until use. Disposable sterile plastic cut-down intravenous catheters

### Table 1. Viable bacterial counts in lungs and leukocyte numbers in bronchoalveolar lavage fluid in mice inoculated with the bacteria

<table>
<thead>
<tr>
<th>Day After Infection</th>
<th>Log$_{10}$ cfu/lung of <em>Pseudomonas aeruginosa</em> S10 (n = 4)</th>
<th>Cell Numbers ($\times 10^4$ cell/ml, n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total cells</td>
</tr>
<tr>
<td>Day 0</td>
<td>0</td>
<td>5.38 ± 0.24</td>
</tr>
<tr>
<td>Day 1</td>
<td>6.0 ± 0.1</td>
<td>102.6 ± 3.5$^a$</td>
</tr>
<tr>
<td>Day 2</td>
<td>5.7 ± 0.2</td>
<td>93.6 ± 22.4$^a$</td>
</tr>
<tr>
<td>Day 3</td>
<td>5.6 ± 0.5</td>
<td>92.8 ± 10.2$^a$</td>
</tr>
<tr>
<td>Day 4</td>
<td>5.9 ± 0.4</td>
<td>105.4 ± 27.1$^a$</td>
</tr>
<tr>
<td>Day 7</td>
<td>5.1 ± 0.6</td>
<td>89.9 ± 10.6$^a$</td>
</tr>
<tr>
<td>Day 14</td>
<td>5.6 ± 0.2</td>
<td>86.5 ± 11.4$^a$</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE. Data in parentheses represent percentage values. $^a P < 0.05$ compared with baseline (day 0).

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**Fig. 1.** Hematoxylin and eosin (H & E) staining of paraffin sections of lung tissues from a representative normal mouse (A) and infected mouse (B, C). Original magnification $\times 100$; n = 2, each. Note the absence of inflammatory cells in the normal lung (A). Note also the severe bronchopneumonia in lung tissue sections of mice at day 2 after inoculation of bacteria (B). At day 14 after infection, note the presence of inflammatory cells around the distal bronchi (C). Alcian blue-periodic acid/Schiff (AB-PAS) staining of paraffin sections of lung tissues from normal (D) and infected mice (E, F). Original magnification $\times 200$; n = 2, each. In the uninfected mouse lung, note the lack of AB-PAS-positive cells (D). In the infected mouse, note the presence of AB-PAS-positive cells in a representative mouse at day 2 (E) and day 14 (F) after inoculation of bacteria. The number of AB-PAS-positive cells and the intensity of AB-PAS staining were very high on day 14.
Enumeration of bacteria in lung tissue. For bacteriological examination, mice were killed at 1, 2, 3, 4, 7, or 14 days after inoculation, and their lungs were dissected out, homogenized, and cultured. The numbers of viable bacteria in the lung were determined quantitatively by serial dilution on Muller-Hinton II agar plate (BBL).

Lung lavage, tissue fixation, and histopathological staining of tissue sections and leukocytes. Lung lavage, tissue fixation, and staining of cells in lung lavage fluid were performed as described previously (17). Briefly, mice were killed 2 and 14 days after inoculation or after 7 days of treatment (clarithromycin or saline). The chest was opened to expose the lungs, and a disposable sterile plastic cut-down intravenous catheter was inserted into the trachea. Bronchoalveolar lavage (BAL) was performed in situ four times sequentially using 1 ml of saline each time, and the recovered fluid fractions were pooled for each animal. Leukocytes in BAL fluid (BALF) samples obtained from each mouse were washed and counted with a hemocytometer. For differential cell counts, cells were centrifuged onto a slide in a table top centrifuge at 1,000 g for 1 min, the slides were stained with May-Giemsa stain, and differential cell counts were performed by counting 100 cells. To prepare lungs for tissue sectioning, the lungs were excised and immersed in 10% phosphate-buffered formalin. Paraffin embedding and tissue staining with Alcian blue (AB) and periodic acid-Schiff (PAS) were performed using standard methods.

Western blot analysis. Lung homogenate was prepared by homogenizing a freshly excised lung in 1 ml of saline. For Western blot analysis, the proteins in the supernatant of the lung homogenate (1,000 g, 5 min) were separated by polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Amersham Pharmacia Biotech, Buckinghamshire, UK). One-hundred micrograms of protein were used for each sample except for the stomach sample, which contained 20 μg of protein. Then, the membranes were blocked with 5% bovine serum albumin in Tris-buffered saline (TBS) and incubated with primary antibody. The membranes were washed with 0.05% Tween 20 in TBS and incubated with secondary antibody for 1 h. After two additional washes, the signal was developed with ECL-Plus (Amersham Pharmacia Biotech). HO1 antibody was generated in chicken using a mouse gastric muc5ac glycoprotein as immunogen and kindly provided by Dr. David Ho at the University of Minnesota at Minneapolis (27). Horseradish peroxidase (HRP)-conjugated goat anti-chicken immunoglobulin G and HRP-conjugated rabbit anti-chicken immunoglobulin G were from Sigma (St. Louis, MO). Antitaxicellular signal-regulated kinase (ERK) 1/2 and antiprophos-ERK1/2 antibody were from Cell Signaling Technology (Beverly, MA).

RT-PCR. Total RNA was isolated using an Isogen (Nippon gene, Tokyo, Japan), and a modified RT-PCR technique was used to determine the mRNA level. Briefly, total RNA was

Table 2. Counts of viable bacteria in the lung and inflammatory cells in the bronchoalveolar lavage fluid of mice treated with saline or clarithromycin

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Log_{10} cfu/lung of Pseudomonas aeruginosa S10 (n = 4)</th>
<th>Cell Numbers (×10^4 cell/ml, n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total cells</td>
<td>Neutrophils</td>
</tr>
<tr>
<td>Saline</td>
<td>5.27 ± 0.26</td>
<td>116.6 ± 29.53</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>5.02 ± 0.24</td>
<td>50.25 ± 14.16†</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE. Data in parentheses represent the percentage of each cell type. *P < 0.05 compared with saline-treated group; †P = 0.089 compared with saline-treated group.
reverse-transcribed into its cDNA by SuperScript First Strand Synthesis System for RT-PCR (GIBCO-BRL, Gaithersburg, MD). Oligonucleotide primers for the PCR were designed according to the published report for mouse muc5ac (39). The PCR conditions used to produce muc5ac by Takara Premix Taq (Takara Shuzo, Otsu, Japan) were 30 cycles of penetration (at 94°C/30 s), annealing (at 55°C/30 s), and extension (at 72°C/30 s) in the presence of 2.5 mg MgCl₂, followed by a final 7-min extension at 72°C. Oligonucleotide primers for hypoxanthine phosphoribosyltransferase (HPRT) were used as an internal control, and the PCR products were separated by electrophoresis through a 2% agarose gel containing ethidium bromide.

Prevention of muc5ac production by inhibitor of intracellular signal transduction. To confirm the involvement of ERK1/2 in muc5ac production, we analyzed prevention of muc5ac by PD-98059 (Promega, Madison, WI), the ERK pathway inhibitor (18). PD-98059 (5 mg) was dissolved in dimethyl sulfoxide (DMSO) and 0.05 ml of dissolved PD-98059 was administrated intravenously 7 days after inoculation of pathogens. Control animals received 0.05 ml of DMSO. Animals were killed a day after treatment, and lungs were excised from mice. Lung homogenate was prepared by homogenizing a freshly excised lung in 1 ml of saline. We analyzed the muc5ac production by Western blotting.

Image and statistical analyses. The signal intensity of each band on Western blots and RT-PCR was analyzed by AlphaImager (Alpha Innotech). Data were expressed as means ± SE. Differences between groups were analyzed for statistical significance using the unpaired t-test. A P value <0.05 denoted the presence of a statistically significant difference.

RESULTS

Viable bacteria in lung and cell counts in BALF. As shown in Table 1, the mean number of viable bacteria recovered from lungs of mice inoculated with bacteria was 10⁵–10⁶ cfu/specimen throughout the observation period. The numbers of total leukocytes, neutrophils, macrophages, and lymphocytes were significantly higher at days 1–14 after inoculation than baseline (day 0). Four animals per group were used for BAL experiment.

Histopathological findings. Histopathological examination of hematoxylin and eosin-stained lung tissue sections showed severe and generalized bronchopneumonia after 2 days of inoculation of P. aeruginosa (Fig. 1B). Lung sections prepared 14 days after inoculation...
showed a population of inflammatory cells around the distal bronchi (Fig. 1C). In uninfected mice lung, AB-PAS-positive cells were not detected (Fig. 1D). In infected mice, however, AB-PAS-positive cells were detected on day 2 (Fig. 1E) and day 14 (Fig. 1F). The number of AB-PAS-positive cells and the intensity of AB-PAS staining in these cells were very high on day 14.

Serial changes in muc5ac production in infected and clarithromycin-treated mice. Western blots of lung muc5ac glycoprotein prepared from mice at 1–14 days after infection are shown in Fig. 2 (data were representative of four independent experiments). Muc5ac glycoprotein was detected on day 1, and the intensity progressively increased from day 4 to day 14. Although 7-day treatment with clarithromycin did not influence the number of viable bacterial colonies in the lung (Table 2), it significantly reduced the number of neutrophils and tended to reduce the number of total cells in BALF (Table 2).

The effect of treatment was further analyzed in AB-PAS-stained sections. Figure 3 showed that treatment with clarithromycin resulted in a decrease in muc5ac-positive cells compared with saline-treated, infected mice. In Western blots of mice treated for 7 days with saline or clarithromycin (n = 5, respectively), quantification of the signal intensity of each band by AlphaImager showed a significant reduction of the signal intensity in PD-98059-treated mice compared with DMSO-treated mice (P < 0.05, Fig. 7).

**DISCUSSION**

Mucus cell hypertrophy and hyperplasia are the hallmarks of many lung diseases, including asthma, chronic bronchitis, and CF (1, 3, 21). Many stimuli, such as bacteria, bacterial LPS, cytokines, elastase, and environmental pollutants, are known to induce mucus cell metaplasia/hyperplasia and hypertrophy (4–6, 8, 9, 23, 24, 28, 30, 36, 39). Mucin glycoconjugate (mucin) is the major macromolecule in the mucus and provides viscoelastic properties to the fluid. Mucin contributes to the natural host defense of the airway by acting as a physical barrier and a medium for segregation of inhaled particles and microorganisms. However, overproduction of mucin enhances chronic infection because mucociliary mechanism is impaired when mucin is produced in large quantities (21).

Although the mechanisms underlying airway hypersecretion in chronic airway disease are not clear at present, infectious or inflammatory events and associated putative mediators may play important roles. Sputum overproduction is one of the most common symptoms of DPB (13). Large quantities of airway secretion interfere with mucociliary clearance of inhaled particles, bacteria, and cellular debris from conducting airway and might cause airway narrowing.

We have established a murine model of chronic respiratory *P. aeruginosa* infection mimicking DPB (41) and used the model to demonstrate in a series of recent studies the effects of macrolide treatment on lymphocyte proliferation (41), biofilm formation (40), and cytotoxicity of three independent experiments. In Western blots of mice treated with DMSO or PD-98059 (n = 5, respectively), quantification of the signal intensity of each band by AlphaImager showed a significant reduction of the signal intensity in PD-98059-treated mice compared with DMSO-treated mice (P < 0.05, Fig. 7).

**Fig. 6.** Involvement of ERK phosphorylation (pERK1/2) in muc5ac expression. We studied whether MAPK are capable of activating muc5ac by examining ERK phosphorylation in immunoblots with the use of phosphospecific antibodies. ERK1/2 phosphorylation was stimulated by infection and was inhibited by clarithromycin (Fig. 6). The MAPK phosphorylation levels indicated are representative of 3 independent experiments. NC, normal control.
tokine production (42). In the present study, muc5ac glycoprotein was detected in lung samples 1 day after inoculation of the microorganism into the lung, and its concentration increased progressively from day 4 to day 14. It is likely that such hypersecretion of the protein contributes to the pathogenic process of chronic pseudomonas infection. This conclusion is based on recent studies showing the important role of mucin-specific adhesion of *P. aeruginosa* in the initial colonization and the role of flagellar cap protein, FlID, in such adhesion process (2).

With regard to the effect of macrolide treatment on mucus secretion, previous studies reported that erythromycin inhibits respiratory glycoconjugate secretion from human airways in vitro (12) and reduced mucin secretion from cultured guinea pig epithelial cells (33). However, to our knowledge, there are no studies that have previously analyzed the effect of macrolides on mucin core proteins. Our results suggest that clarithromycin, a macrolide antimicrobial agent, inhibited muc5ac production in chronic respiratory infection in vivo.

How does clarithromycin inhibit mucus overproduction? One possible mechanism of action is direct inhibition of mucus secretion. Mucin gene expression is regulated by transcriptional activating proteins such as nuclear factor-κB (NF-κB) and activating protein-1 (22, 34). Previous studies have demonstrated that clarithromycin modulates tumor necrosis factor-α-induced NF-κB activation in U-937, Jurkat, and A549 cells and peripheral blood mononuclear cells (PBMC) and also modulates staphylococcal enterotoxin-induced NF-κB activation in PBMC (15). Van Seuningen et al. (35) reported that LPS upregulated MUC5AC in lung epithelium cells through the Ras/MAPK/pp90rsk/NF-κB pathway. We showed that ERK1/2 (one of the MAPK) phosphorylation was stimulated by infection and was inhibited by clarithromycin. And prevention of muc5ac production by the ERK pathway inhibitor strongly suggests the involvement of ERK in muc5ac production. These results support the idea that macrolide inhibits mucin gene expression through the modulation of intracellular signal transduction.

In conclusion, our results suggest that the inhibitory effects of macrolide on mucus hypersecretion may explain the clinical improvement seen in patients treated with these antimicrobial agents.

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REFERENCES


