BEHAVIOR OF MUCUS GLYCOPROTEINS OF TRACHEAL SECRETORY CELLS FOLLOWING L-CYSTEINE METHYL ESTER TREATMENT

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The effects of L-cysteine methyl ester on behavior of mucus glycoproteins in canine tracheal secretory cells were investigated histologically and histochemically. Low concentrations (10^{-7} and 10^{-6} M) of L-cysteine methyl ester produced, as histological changes, decreases in the total goblet cell number and the thickness of the acini of submucosal glands, and an increase in the acinar inner diameter. On the other hand, L-cysteine methyl ester at 10^{-5} and 10^{-4} M produced an increase in the thickness of the acini and a slight decrease in the acinar inner diameter. Both the total acid glycoprotein and sulphated glycoprotein contents in glandular cells markedly decreased, whereas the neutral glycoprotein content in the cells increased following L-cysteine methyl ester treatment in a concentration-dependent way. Total saccharide and protein concentrations in the incubation fluid increased with 10^{-7} and 10^{-6} M, but decreased with 10^{-5} and 10^{-4} M L-cysteine methyl ester. *N*-Acetylhexosamine concentration in the bathing fluid increased at 10^{-7} and 10^{-6} M of the agent. These findings suggest that low concentrations of L-cysteine methyl ester stimulate secretory activities of tracheal secretory cells, while at high concentrations the agent stimulates synthesis of mucus glycoproteins in the submucosal glands. Additionally, it is suggested that L-cysteine methyl ester has a marked viscosity lowering effect on the mucus in the secretory cells.

Keywords—L-cysteine methyl ester; trachea; goblet cell; submucosal gland; secretory activity; mucus glycoproteins; histochemical study

INTRODUCTION

The mucin component of airway secretions mainly consists of high molecular glycoproteins which are synthesized and secreted by both goblet cells in the airway epithelium and submucosal glands in the lamina propria mucosae.¹⁾ The acid glycoprotein (AGP) content of mucus glycoproteins is a principal factor in determining the viscoelasticity of the secretions.²⁻⁴⁾

L-Cysteine methyl ester is one of the cysteine-type mucolytics which are widely used in patients with pulmonary diseases. A few studies on the effects of L-cysteine methyl ester on sputum viscosity⁵⁾ and on respiratory tract fluid (RTF) output⁶⁾ have been reported. Effects of this agent on secretory activity of airway secretory cells including on behavior of mucus glycoproteins in these cells are, however, poorly understood.

In the present study, we investigated the

secretagogic and mucolytic effects of L-cysteine methyl ester on canine tracheal secretory cells histologically and histochemically.

MATERIALS AND METHODS

Drug Treatment—Adult male mongrel dogs weighing between 7 and 10 kg were anesthetized with sodium pentobarbital (30 mg/kg *i. v.*), and the trachea, about 10 cm in length from the larynx, was excised.

The tracheal segments (about 2 cm long) were preincubated for 30 min in an organ bath containing 24.5 ml of Hanks' solution. The bathing solution was oxygenated with a 95% O₂–5% CO₂ mixture and maintained at $37 \pm 1^\circ\text{C}$. After preincubation, the tracheal segment was treated for 30 min with L-cysteine methyl ester hydrochloride (Kissei) at the concentration being tested (10^{-7} to 10^{-4} M). Then the tissue was fixed, and the

incubation fluid was also stored at -20°C until the total saccharide, *N*-acetylhexosamine and protein in the fluid were analyzed.

Preparation of Tissues—The drug-treated tracheal tissues were fixed, by stages, with 1.25 and 2.5% glutaraldehyde in 0.2 M Millonig's phosphate buffer (pH 7.4) below 4°C for 2 h. The tissues were cut into small pieces (about $5\text{ mm} \times 5\text{ mm}$), and they were then fixed again with a mixture of 2% paraformaldehyde and 2.3% glutaraldehyde in 0.2 M Millonig's phosphate buffer (pH 7.4) below 4°C for 15 h.

After fixation, the tissues were dehydrated with ethyl alcohol, infiltrated with paraffin wax, and sectioned in $4\text{ }\mu\text{m}$ slices. The tissue slices were stained with a combination of alcian blue (AB) at pH 2.5 or pH 1.0 and periodic acid-Schiff (PAS) (designated as AB (pH 2.5)/PAS or AB (pH 1.0)/PAS).

Quantitation of Histological and Histochemical Changes—To assess the effect of L-cysteine methyl ester, five tracheas were used for each concentration. Fifty slices were made from a trachea treated with the agent. Ten out of the 50 slices,

chosen at random, were stained and photographed at 150 fold magnification.

Histological and histochemical changes in the submucosal glands and goblet cells were evaluated quantitatively in the following manner.

(i) Histological Parameters⁷⁻¹¹: Submucosal glands: Reid index (GWR), acinar outer (A^O) and inner diameter (A^I), thickness of acinus ($A^O - A^I$) and the ratio of acinar inner diameter to the tracheal wall thickness ($A^I\text{WR}$). Goblet cells: number of goblet cells stained positively within a range of $400\text{ }\mu\text{m}$ length of the tracheal

FIG. 1. Schematic Illustration of Tracheal Tissues and Histological Parameters used in This Study

A^I : acinar inner diameter, A^O : acinar outer diameter, and GL: gland layer. Reid index (GWR): the ratio of GL thickness to wall thickness; the wall thickness is the tissue thickness between the basement membrane of the epithelium and the inner side of the cartilage. $A^I\text{WR}$: the ratio of A^I to wall thickness.

FIG. 2. The Canine Tracheal Epithelium stained with AB (pH 2.5)/PAS at $300\times$ Magnification

A; the control group and B; L-cysteine methyl ester (10^{-4} M)-treated group. GC; goblet cell.

epithelium (Fig. 1).

(ii) Histochemical Parameters⁸⁻¹⁴: With the AB (pH 2.5)/PAS procedure, all AGP stain blue (stain index blue (B)) or purple (stain index purple (P)), and neutral glycoproteins (NGP) stain red (stain index red (R)). With the AB (pH 1.0)/PAS procedure, only sulphated glycoproteins (SGP) of the AGP stain blue (stain index B) or purple (stain index P).

Analysis of Macromolecular Components in the Incubation Fluid — The total saccharide concentration in the incubation fluid was determined by the Anthrone method.¹⁵ The protein concentration was determined by the Folin-phenol method.^{16,17} The *N*-acetylhexosamine concentration was estimated according to the method of Reissig *et al.*¹⁸

RESULTS

Typical photomicrographs of goblet cells and submucosal glands in the trachea treated with L-cysteine methyl ester are shown in Fig. 2 and 3.

Effects of L-Cysteine Methyl Ester on Goblet Cells

Total number of goblet cells stained positively with AB (pH 2.5)/PAS was 20.3 ± 2.7 ($N=5$, mean $\pm$ S.E.) in the control group. L-Cysteine methyl ester caused a slight decrease in the number to 16.2 ± 2.4 and 17.9 ± 1.4 at concentrations of 10^{-7} and 10^{-6} M, respectively (Fig. 4). The number of total AGP-containing goblet cells, which showed a stain index B & P, was 15.0 ± 2.2 in the control group. After an application of L-cysteine methyl ester, it was significantly decreased to 6.5 ± 2.3 at 10^{-7} M, 7.7 ± 1.4 at 10^{-6} M, 8.2 ± 1.1 at 10^{-5} M and 7.7 ± 0.9 at 10^{-4} M. On the other hand, the number of NGP-containing cells, which showed a stain index R, was significantly increased in a concentration-dependent way. (Fig. 4).

The changes in the number of SGP-containing goblet cells, which showed a stain index B & P with AB (pH 1.0)/PAS, are shown in Fig. 5. L-Cysteine methyl ester significantly reduced the SGP-containing goblet cell number from 16.1 ± 1.4 (control) to 8.8 ± 2.5 (10^{-7} M), 8.3 ± 1.3 (10^{-6} M), 8.8 ± 2.4 (10^{-5} M) and 7.8 ± 1.5 (10^{-4} M).

FIG. 3. The Canine Tracheal Submucosa stained with AB (pH 2.5)/PAS at 300 $\times$ Magnification A; the control group, B; L-cysteine methyl ester (10^{-7} M)-treated group and C; L-cysteine methyl ester (10^{-4} M)-treated group. SG; submucosal gland.

Effects of L-Cysteine Methyl Ester on Submucosal Glands

The acinar outer diameter (A^O) of submucosal glands was $32.1 \pm 1.9 \mu\text{m}$ ($N=5$, mean $\pm$ S.E.) in the control group. After L-cysteine methyl ester treatment at concentrations of 10^{-7} and 10^{-6} M, the diameters showed a slight increase to 35.4 ± 1.9 and $35.1 \pm 2.5 \mu\text{m}$, respectively. On the other hand, the groups treated with the agent at the concentrations of 10^{-5} and 10^{-4} M showed no difference in the diameter as compared with the control value.

L-Cysteine methyl ester at concentrations of 10^{-7} and 10^{-6} M caused an increase in the acinar inner diameter (A^I) from 9.9 ± 1.1 (control) to 15.7 ± 1.2 ($p < 0.01$) and $13.5 \pm 1.3 \mu\text{m}$, respectively, whereas at 10^{-4} M A^I was rather decreased to $6.8 \pm 1.2 \mu\text{m}$.

FIG. 4. Number of Goblet Cells stained with AB (pH 2.5)/PAS per 400 μm Length of the Tracheal Epithelium

Each curve represents the number of goblet cells containing total (—○—), acid (—●—; stain index B & P) or neutral glycoprotein (—□—; stain index R). Each value represents the mean $\pm$ S.E. of 5 experiments (Value for each experiment is the average determined from 5 slices).

Significant at a) $p < 0.05$ and b) $p < 0.01$ as compared to the control.

The changes in the thickness of acinus ($A^O - A^I$) and the ratio of acinar inner diameter of gland to wall thickness (A^I/WR) are shown in Fig. 6. A slight decrease in the thickness was observed when L-cysteine methyl ester was applied at low concentrations. On the other hand, the thickness tended to be increased at 10^{-5} and 10^{-4} M. A^I/WR was increased by L-cysteine methyl ester treatment at concentrations of 10^{-7} and 10^{-6} M. Especially, a marked change was seen at 10^{-7} M.

After L-cysteine methyl ester treatment, the increment percentages of Reid index (GWR) were 12.3% at 10^{-7} M, 10.0 at 10^{-6} M, 4.8 at 10^{-5} M and 4.4% at 10^{-4} M.

The changes in the stain index of submucosal glandular cells with AB (pH 2.5)/PAS after an application of L-cysteine methyl ester are shown in Fig. 7. The total number of AGP-containing glandular cells, which showed a stain index B & P with AB (pH 2.5)/PAS, was markedly reduced, while the number of NGP-containing cells,

FIG. 5. Number of Goblet Cells stained with AB (pH 1.0)/PAS per 400 μm Length of the Tracheal Epithelium

The curve represents the number of goblet cells containing sulphated glycoprotein (stain index B & P). Other explanations are as in Fig. 4.

which showed a stain index R, was increased in a concentration-related manner. The number of SGP-containing glandular cells, which showed a stain index B & P with AB (pH 1.0)/PAS, was markedly decreased from 89.2% (control) of the total number of glandular cells evaluated (NG=92) to 52.3% (10^{-7} M; NG=123), 50.8% (10^{-6} M; NG=89), 40.3% (10^{-5} M; NG=101) and 32.4% (10^{-4} M; NG=98), like the case of total AGP-containing cells.

Changes in Macromolecular Components in the Incubation Fluid caused by L-Cysteine Methyl Ester

The changes in total saccharide, *N*-acetylhexosamine and protein concentrations in the incubation fluid following L-cysteine methyl ester treatment are shown in Fig. 8. *N*-Acetylhexosamine concentration in the fluid was 2.15 ± 0.71 mg/24.5 ml ($N=5$, mean $\pm$ S.E.) in the control group. The concentrations were

increased to 4.96 ± 0.11 ($p < 0.01$) and 3.83 ± 0.61 at 10^{-7} and 10^{-6} M L-cysteine methyl ester, respectively, while at 10^{-5} and 10^{-4} M it approximated to the control value.

Total saccharide and protein concentrations in the fluid rose in the presence of L-cysteine methyl ester at 10^{-7} and 10^{-6} M, but were decreased at 10^{-5} M and 10^{-4} M.

DISCUSSION

Sheffner¹⁹⁾ and Sheffner *et al.*²⁰⁾ found that cysteine derivatives including *N*-acetyl-L-cysteine decrease mucus viscosity *in vitro*. According to them, compounds containing a terminal-SH group can break the disulfide bridges in a mucus glycoprotein molecule.

L-Cysteine methyl ester is also one of the SH-containing amino acids, the structure being $\text{HS-CH}_2\text{-CH(NH}_2\text{)-COOCH}_3$. Saia *et al.*⁵⁾ reported

FIG. 6. Changes in Thickness and A^IWR of the Acinus in Tracheal Submucosal Gland

Each column represents the mean $\pm$ S.E. of 5 experiments (Value for each experiment is the average determined from 10 slices).

Significant at a) $p < 0.05$ as compared to the control.

that this agent decreased, in *in vitro* experiments, the viscosity of both sputum of patients with chronic catarrhal bronchitis and gastric mucin. Recently, it was shown that L-cysteine methyl ester caused a slight increase in the RTF output at doses of 30 and 100 mg/kg *p.o.* and a decrease in the output at a dose of 300 mg/kg *p.o.* in anesthetized dogs.⁶⁾ However, the effects of this agent on the activities of tracheal secretory cells including behavior of mucus glycoproteins of the cells have not yet been studied in detail.

In the present study, it was found that low concentrations of L-cysteine methyl ester caused a decrease in the total number of goblet cells which contain glycoproteins in plenty and additionally, a decrease in acinar cell thickness and an increase

in A^IWR in submucosal glands. These findings suggest that L-cysteine methyl ester at low concentrations may stimulate discharge of mucus glycoproteins from both goblet cells and submucosal glands. This is supported by the finding that increases in total saccharide, *N*-acetylhexosamine and protein concentrations in the incubation medium were observed after an application of low concentrations of L-cysteine methyl ester.

Quevauller *et al.*^{21,22)} and Huyen²³⁾ reported that large doses of *S*-carboxymethylcysteine had a stimulant effect on synthesis of mucus glycoproteins in goblet cells in SO₂-induced bronchitic rats. In the present study, L-cysteine methyl ester at high concentrations induced slight histological

FIG. 7. Changes in Stain Indexes of Tracheal Submucosal Glandular Cells with AB (pH 2.5)/PAS. Each column represents the percentage of glandular cells containing acid (hatched; stain index B and dotted; stain index P) and neutral glycoproteins (white; stain index R). The numbers of glandular cells evaluated were 109 (control), 190 (L-cysteine methyl ester 10⁻⁷ M), 152(10⁻⁶ M), 174(10⁻⁵ M) and 103(10⁻⁴ M), respectively.

FIG. 8. Changes in Total Saccharide, *N*-Acetylhexosamine and Protein Concentrations in the Incubation Fluid. Each curve represents total saccharide (—○—), *N*-acetylhexosamine (—●—) or protein (—□—) concentration. Each value represents the mean $\pm$ S.E. of 5 experiments. Significant at a) $p < 0.05$ and b) $p < 0.01$ as compared to the control. H.S.: Hanks' solution.

changes such as a decrease in A^I and an increase in glandular cell thickness. Also, an enhanced incorporation of saccharides in the medium into the incubated trachea occurred with high concentrations of L-cysteine methyl ester. It is thus considered that high concentrations of L-cysteine methyl ester may stimulate synthesis of mucus glycoproteins in submucosal glandular cells, rather than stimulate discharge of mucus. That is, the threshold concentration of L-cysteine methyl ester needed to stimulate synthesis of mucus glycoproteins should be higher than that required to stimulate discharge of mucus glycoproteins.

After L-cysteine methyl ester treatment, the number of total AGP- and SGP-containing cells in both the epithelium and the submucosal layer were markedly decreased. On the other hand, the number of NGP-containing cells was increased. These histochemical changes suggest that this agent may alter the composition of mucus glycoproteins in secretory granules of both goblet cells and submucosal glands. Another possibility is that L-cysteine methyl ester may dissolve the high molecular AGP through a mucolytic action inside the cells, being suggested from the fact that AB colors blue only when one dye molecule combines with several polyanion sites of an AGP molecule.^{24,25)}

In summary, we consider that the expectorant action of L-cysteine methyl ester may result from both promoting the secretory activity of airway secretory cells and reducing the viscosity of glycoproteins of mucus granules.

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