Current status of mucus drug research: Strategies and problems

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1. Introduction

A common finding in airway diseases is a disturbance of the normal mucociliary clearance due to a hyperproduction of mucus and a modification in its physicochemical characteristics. Drugs active on airway secretion have been proposed and used to cleanse and clear the respiratory tract for many centuries and in many countries. On the basis of the mechanism of the action, the mucoactive drugs were classified into several groups [1]. Some mucoactive drugs possess direct effects on the production of airway secretions or on changes in their composition, resulting in increased effectiveness of mucociliary clearance. Other mucoactive drugs do not have a specific action on mucus, but produce a general benefit on airway structure and function, which secondarily lead to correction of the pathophysiologic mechanisms that result in abnormal secretions. However, since many drugs have overlapping effects, it is difficult to simply classify these drugs into some groups based on their major actions. For example, it is well known that ambroxol stimulates the formation and release of pulmonary surfactant by alveolar type II cells [2]. However, recent reports indicated that ambroxol as well as N-acetyl-L-cysteine and carboxymethylcysteine could sufficiently enhance the antioxidant defense mechanisms in lung tissues and could act as lung lipid antioxidants. [3-9]. In addition, ambroxol inhibited the chemotactic response and spontaneous migration of human polymorphonuclear leukocytes (PMNLs) [10-12], and attenuated the production of interleukin-1 and tumor necrosis factor by human mononuclear cells [13]. In *in vivo* studies, ambroxol diminished the bleomycin-induced lung injury in rats [14], and decreased their mortality after an administration of paraquat, a herbicide that generates reactive oxygen species [15]. Accordingly, ambroxol as a mucoactive drug fundamentally has an anti-inflammatory action. Taken together with previous findings of mucoactive drugs, it seems to us that the anti-oxidant effect, as a crucial action to exert their effects against airway diseases, is a common property in mucoactive drugs. In the light of this

idea, we must use specific experimental models to simulate pharmacological events in airway inflammation. Nowadays, the development of new techniques has made it possible to identify and measure the mucus components, to measure the rheological parameters more accurately, and to evaluate mucociliary clearance precisely in animals and humans. Therefore, from various points of view, we have evaluated mucoactive drugs with modifications of methodological approaches to reflect actions in inflammatory states for two decades. We will introduce here our overall methods to study many parameters involved in airway clearance, and some mucoactive drugs we have studied recently.

2. Screening systems for mucoactive drugs

2.1. *in vivo* screening systems for airway secretion in inflammation

There are several reports on experimental models of airway inflammation. Of the reports, we used a SO_2-exposed model as an *in vivo* screening system for airway secretion in inflammation, because SO_2 mainly causes airway epithelial damages like features of bronchitis. There are two convenient methods to study airway secretion; the Perry and Boyd method and broncho-alveolar lavage method.

The Perry and Boyd method has been used extensively to study the effects of sympathetic or parasympathetic agents and the effects of many kinds of mucoactive drugs. In our previous studies, the mucus production of rabbits with subacute bronchitis induced by a long-term exposure (5 weeks to 3 months) to SO_2 (50 to 300 ppm) has been determined with this method. We determined sugar, protein and phospholipid contents in airway secretions from normal and bronchitic rabbits and evaluated many mucoactive drugs on airway secretion [16-21]. The majority of sugars in mucins are composed of fucose, galactose, N-acetylgalactosamine, N-acetylglucosamine and N-acetylneuraminic acid. We found that the sugars were increased in airway secretions of SO_2-exposed rabbits. Especially, the increases of galactose and N-acetylglucosamine suggest that airway secretions from SO_2-exposed rabbits are composed of long chains of sugars in mucins, resulting in viscous property of the secretions similar to the sputa from bronchitic patients. The reason is that these two sugars are major components of elongated sugars in mucins, which is based on sugar structure of mucin.

Broncho-alveolar lavage methods were also applied to study airway secretions. The merit of this method is to collect many components enough amount to study in detail. For example, we examined an influence of long term exposure to SO_2 on the pulmonary surfactant by the method. [22]. Recently, in order to investigate mucin production in pathological states, we made monoclonal antibodies (4H6, 2D11) against the mucins from broncho-alveolar lavages of hamsters with bronchitis caused by SO_2 exposure [23]. In the

immunohistochemical studies, the antibodies recognized the mucins secreted into lumen, but not the mucins stored in goblet cells nor submucosal gland mucous cells. The ELISA has shown that the antibodies react with some mucins from hamster intestine and swine stomach, and broncho-alveolar lavages of rats, guinea-pigs, dogs and human. However, the antibodies did not recognize bovine submaxillary gland mucins nor proteoglycans. Therefore, the ELISA using the antibodies could be available to quantify airway mucin production in future mucoactive drugs screening.

2.2. *in vitro* screening systems for airway secretion in inflammation

For drug evaluation, we used two kinds of *in vitro* models to evaluate mucoactive drugs. To investigate mucus secretions as a major component of gel layer of airway secretions, we have used culture systems of hamster tracheal epithelial cells or human pulmonary mucoepidermoid carcinoma cell lines (NCI-H292). To investigate pulmonary surfactant as a major component of sol layer, we have used primary culture of rat alveolar type II cells.

Kim et al. have reported that hamster tracheal epithelial cells in culture are morphologically and biochemically similar to goblet cells [24, 25]. Using this system, it has been reported that the secretion of high molecular weight glycoconjugates (HMWG), a marker of mucus, is influenced not by pharmacological agents such as acetylcholine and histamine, but by alteration of medium pH, cationic proteases, alteration of medium osmolarity, mechanical strain of cells and ATP [26-30]. We also found that protein kinase C was involved in HMWG secretion in hamster tracheal epithelial cells in culture [31]. Recently, we have found that adrenergic agonists suppressed HMWG secretion in hamster tracheal epithelial cells.

To simulate the inflammatory states, we have used co-culture systems of mucus cells with polymorphonuclear leukocytes (PMNLs) activated by several stimuli [32, 33]. Abnormal and excessive mucus secretion is a characteristic feature of many chronic inflammatory lung diseases accompanied by the influx of PMNLs into the airway and by the release of substance P from the peripheral endings of primary sensory neurons. We examined whether PMNLs activated by substance P (10 μM) can affect the secretion of HMWG from cultured hamster tracheal epithelial cells. We measured both the released and the cell-associated HMWG. Substance P-activated PMNLs (10^6 cells/ml) reduced the amount of cell-associated HMWG to 76% of the control level, but did not affect the amount of the released HMWG. The reduction of the amount of cell-associated HMWG was inhibited by ONO-5046, a specific elastase inhibitor. In addition, the HMWG was digested by the activated PMNLs. These findings suggested that substance P stimulated the release of the cell-associated HMWG and degraded the released HMWG from cultured hamster tracheal epithelial cells through PMNLs

activation. As suggested in many reports, we indicated that neutrophil elastase may be a crucial mediator to induce mucus secretion in inflammation.

Pulmonary surfactant, which is composed of phospholipids and apoproteins and is mainly produced in alveolar type II cells, lowers the surface tension at the air-liquid interface in the lung and provides for alveolar stability. De Sanctis clearly demonstrated that, in addition to the vital role, surfactants are also important in airway mucociliary clearance [34]. Several studies have suggested that the presence of phospholipids in the airways may modify the clearance of mucus [35-40]. The existence of surfactant films has been demonstrated in the airways of several species by electron microscopy [37, 41], and by *in situ* surface tension measurement [37, 42]. We also confirmed a protective effect of surface active phospholipids on an acid-inducing inhibition of mucociliary transport in pigeons [43].

Taken together with many reports on pulmonary surfactant in mucociliary clearance, it is reasonable to study the effects of mucoactive drugs on pulmonary surfactant secretion in alveolar type II cells. Secretion of phosphatidylcholine, a major surfactant phospholipid, has been shown to be influenced by a variety of physiological and pharmacological agents in alveolar type II cells [44, 45]. We also found that both β_1- and β_2-adrenoceptor subtypes mediated phosphatidylcholine secretion [46] and confirmed that both β_1- and β_2-adrenoceptor genes were expressed in rat alveolar type II cells [47].

To simulate inflammation states, we have used co-culture systems of alveolar type II cells with activated PMNLs or eosinophils [48, 49]. Activated PMNLs and eosinophils in airway epithelium are thought to be involved in the pathogenesis of many airway diseases. PMNLs or eosinophils activated by opsonized zymosan caused a significant increase in phosphatidylcholine secretion. Pretreatment of the culture with the combination of superoxide dismutase and catalase reduced the increase in phosphatidylcholine secretion. These results suggested that activated PMNLs and eosinophils stimulated the secretion of pulmonary surfactant, and that the stimulation was partly mediated by oxygen radicals. These systems may be useful to assay the anti-oxidant effects of mucoactive drugs.

2.3. Mucociliary transport in inflammation

Mucociliary clearance is an important pulmonary defense mechanism that serves to remove inhaled substances from the lung [50]. The mucociliary function is depressed by a variety of water-soluble atmospheric pollutants such as SO_2 and NO_2 [50]. The techniques for *in vivo* measurement of mucus transport rates involve the placement of an optically, radiographically, or scintigraphically detectable solid or liquid marker on the mucosa [51].

To simulate inflammatory states, we have used pigeons and quails for the evaluation of drugs on mucociliary transport. A major reason for the use of birds is based on histological findings and biochemical study of broncho-alveolar lavages. The histological features are similar to airway inflammation states. There are many proliferated goblet cells and submucosal glands in tracheal epithelium. Furthermore, in broncho-alveolar lavages of birds, extremely higher content of fucose, a typical sugar in mucins was found. So far, we examined the effects of many mucoactive drugs on mucociliary clearance in birds [16, 20, 43, 52-58]. For example, we found that inhalation of bromhexine, classified as a mucolytic agent, increased mucociliary transport in quails. Recently, we investigated the effect of leukotriene D_4 (LTD_4) on mucociliary transport in quails [59]. Topical application of LTD_4 (0.2 - 2 ng) to tracheal mucosa dose-dependently increased mucociliary transport 5 or 10 min after the application. Forty minutes after the application of 2 ng of LTD_4, mucociliary transport was decreased to about 84% of that in the control group. Both the transient increase and the subsequent decrease were blocked by ONO-1078 (0.03 - 3 mg/kg, i.m.), a specific LTD_4 receptor antagonist. These results suggested that LTD_4 possessed a biphasic effect on tracheal mucociliary transport through leukotriene receptors. Moreover, we found that topical application of histamine (1 pmol) to tracheal mucosa prominently decreased mucociliary transport 5 or 10 min after the application. The inhibitory effect was restored by the anti-allergic drug.

In preliminary experiments, we found that sphingomyelin-rich lipid fractions from sputa suppressed mucociliary transport in trachea. Although the mechanism of action remains unclear, the finding suggests that we have to consider composition of phospholipids in sputa as inhibitory factors of mucociliary clearance.

3. Current mucoactive drugs

We have evaluated several mucoactive drugs for two decades. Of the drugs, we will introduce here the following drugs with anti-inflammatory properties: sodium aceneuramate, glucocorticoids, Chinese traditional medicines, new cysteine derivatives.

3.1. Sodium aceneuramate (N-acetylneuraminic acid (NANA) sodium salt)

Although participation of sialic acids, mainly NANA, in many biological and pathological processes has been well documented [60], there are only a few studies concerning the significance of the sialic acid in the airway [61]. We found that the sputa of bronchitic rabbits contained much higher levels of both free and bound NANA than the airway secretions of normal rabbits by a selected ion monitoring technique [62, 63]. According to our preliminary experiments, NANA

concentrations in the sputa of patients with chronic bronchitis (free, 15-204 μg/ml; bound, 276-1298 μg/ml) were also apparently higher than those in the broncho-alveolar lavages of healthy subjects. In addition, we have reported that inhalation of NANA repaired inflammation in the airway, resulting in bronchitic rabbits to produce sputa with low viscosity like normal airway secretions [21, 63]. Further, NANA, but not lactose, dose-dependently protected the mucociliary transport impaired by cigarette smoke [55]. The results suggest that NANA may participate the defense mechanism in the airway against irritant gases. In addition, we studied the *in-vivo* anti-allergic effect of NANA in guinea-pigs passively sensitized with anti-ovalbumin rabbit serum [64]. NANA inhibited bronchial anaphylaxis and the release of histamine into broncho-alveolar lavages. NANA dose-dependently attenuated heterologous passive cutaneous anaphylaxis and hemorrhaging in the passive Arthus reaction. Interestingly, NANA did not inhibit the release of histamine from sensitized minced lung tissues *in vitro*. The clinical observations [65, 66] that NANA (sodium aceneuramate) is an effective inhalant expectorant seems to support our findings. We administered sodium aceneuramate (2 mg, twice a day for 7 days) by inhalation to 10 patients with bronchiectasis, chronic bronchitis, diffuse panbronchiolitis and other respiratory diseases that cause sputa [66]. We found that sodium aceneuramate improved subjective symptoms, such as the number of expectoration of sputa, and sticking sensation of sputa. The viscosity of sputa was increased in 4 patients and the ratio of disaturated phosphatidylcholine (DSPC)/phosphatidylcholine (PC) was increased in 5 patients but protein contents were decreased in 4 patients whose subjective symptoms were improved. Sodium aceneuramate did not cause any side effects and any abnormal laboratory findings. Therefore, it was possible that sodium aceneuramate improved the subjective respiratory symptoms due to sputa.

3.2. Glucocorticoids

Glucocorticoids are first-choice drugs in the management of the inflammatory process seen in asthma. Although current pharmacological approaches to airway mucus production are limited, glucocorticoids seem to be the most effective among a few useful drugs. However, there are a few studies documenting the benefits of glucocorticoid therapy on the mucociliary clearance and the excessive production of airway mucus. Systemic glucocorticoids ameliorated bronchial obstruction and facilitated expectoration in patients with asthma and chronic bronchitis, although they did not alter sputum viscosity [67]. Direct exposure of the bronchial mucosa to prednisolone resulted in mild cilioexcitation [68], whereas the topical beclomethasone had no effect on mucociliary transport in conscious sheep [67]. Thus, the contribution of glucocorticoids to mucociliary transport remains unclear. We previously examined the effect of corticosterone on

tracheal mucociliary transport in pigeons [57]. Corticosterone (5.0 mg/kg) significantly increased the mucociliary transport rate. Metyrapone, an adrenal 11-β steroid hydroxylase inhibitor, significantly decreased the mucociliary transport rate, and the inhibitory action was blocked by 1.0 mg/kg of corticosterone. The findings suggested that glucocorticoids modulated the mucociliary clearance, especially under some diseases associated with a decreased level of endogenous corticosteroids.

As for effects on mucus production, several reports have shown that glucocorticoids directly reduce submucosal gland secretions [69-72] without a significant alteration in the synthesis of mucins [71]. Recently we tried to clarify the effect of dexamethasone on mucus production and mucin gene expression in a human pulmonary mucoepidermoid carcinoma cell line (NCI-H292). Dexamethasone (10^{-8} - 10^{-6} M) significantly suppressed the basal production of [^{3}H]glucosamine- or [^{3}H]serine-labeled HMWG in NCI-H292 cells. To examine the effect on mucin gene expression, we have selected MUC-2 and MUC-5 out of eight mucin genes (MUC-1 - MUC-8) [73-85], because MUC-2 was expressed in the airways of patients with inflammatory airway disorders, such as cystic fibrosis and chronic bronchitis [86-89], and because MUC-5 was cloned from the human airway [82]. In Northern blot analysis, dexamethasone (10^{-9} - 10^{-7} M) attenuated steady-state mRNA levels of MUC-2 and MUC-5 mucin genes. Thus, we concluded that dexamethasone suppressed the basal production of HMWG and decreased steady-state mRNA levels of mucin genes in airway mucus-producing cancer cells.

3.3. Chinese traditional medicines

There is an increasing usage of Chinese traditional herbal medicines in clinics and hospitals, because the Chinese medicines tend to have moderate side effects and sometimes produce remarkable efficacy. In order to renormalize overall defects in airway disorders, Chinese medicines may be adequate drugs, because the medicines are composed of various herbs with weak, but ubiquitous pharmacological activities.

Qing-Fei-Tang, consisting of 16 herbs, was described in 'Wang Bin Hui Chun,' the medical literature published in 1587 in China. *Qing-Fei-Tang* has been clinically applied to treat chronic obstructive pulmonary diseases with severe cough and sputum. *Qing-Fei-Tang* was also effective for the treatment of the bronchitis with an asthmatic attack-like symptom [90]. In this patient, the abnormally elevated chemiluminescence of oxygen radicals in leukocytes was normalized as symptoms improved through 5 weeks' administration of *Qing-Fei-Tang*. Our previous study showed that *Qing-Fei-Tang* inhibited the release of slow reacting substance of anaphylaxis from passively sensitized guinea-pig lung after antigen challenge [91]. *Qing-Fei-Tang* also suppressed the

chemiluminescence of oxygen radicals, when healthy human leukocytes were stimulated by opsonized zymosan. In normal rabbits, *Qing-Fei-Tang* increased the output volume and fatty acid contents in airway secretions. In the bronchitic rabbits, 6 weeks' administration of *Qing-Fei-Tang* restored the decreased amount of saturated fatty acid in the sputa, and histological examinations revealed an amelioration of the inflammation of lung tissues. In pigeons, *Qing-Fei-Tang* facilitated tracheal mucociliary transport. Accordingly, *Qing-Fei-Tang* seems to exert effectiveness via its multiple mechanisms.

As another Chinese traditional medicine, we have investigated *Mai-men-Dong-Tang*, consisting of 6 herbs, *Ophiopogonis Tuber*, *Pinelliae Tuber*, *Zizyphi Fructus*, *Glycyrrhizae Radix*, *Ginseng Radix* and *Oryzae Fructus*. *Mai-men-Dong-Tang* has been used for the treatment of bronchitis and pharyngitis accompanying severe dry cough. We found that unlike codeine, *Mai-men-Dong-Tang* had a notable antitussive activity against the cough associated with bronchitis and the cough increased by angiotensin-converting enzyme inhibitors [92]. Recently, we found that in alveolar type II cells, *Mai-men-Dong-Tang* attenuated phosphatidylcholine secretion increased by oxygen radicals from activated PMNLs. In addition, we found that *Mai-men-Dong-Tang* by itself slightly stimulated phosphatidylcholine secretion and increased β_1-adrenoceptor gene expression in rat alveolar type II cells. Although the mechanism of action remains unclear, the effect may contribute to the effectiveness on chronic airway diseases.

3.4. New cysteine derivatives

We are developing a new cysteine derivative, S-(3-hydroxypropyl)-L-cysteine (SS320A), as a new mucoactive drug [93]. In rabbits, SS320A significantly increased pulmonary secretion of the marker dye, indicating bronchosecretagogue activity. In addition, SS320A increased the volume of airway secretions in normal rabbits collected by the Perry and Boyd method. SS320A (10^{-2} M) did not influence the rheological properties of the pig gastric mucin in vitro. SS320A (500 mg/kg/day, p.o., 2 weeks) restored the decreased content of free sialic acid in broncho-alveolar lavages in bronchitic animals made by long term SO_2 exposure. SS320A inhibited the hyperplasia of goblet cells in airway epithelium caused by isoproterenol (0.05 mg/kg, i.p.). SS320A (500 mg/kg, p.o.) did not affect the normal tracheal mucociliary transport in quails, while inhalation of SS320A dose-dependently restored the mucociliary transport impaired by a cigarette smoke exposure. The results suggest that SS320A possesses mucoactive and mucoregulatory activity.

Erdosteine, dl-S-{2[N-3-(2-oxotetrahydrothienyl)acetamine]} thioglycolic acid (under development in Japan as KW-9144) is a novel thiol derivative endowed with mucolytic, mucomodulatory and free radical scavenging properties, and without gastric adverse effects unlike

other cysteine derivatives [94]. We found that erdosteine (600 mg/kg, p.o.) significantly promoted mucociliary transport in quails and suppressed capsaicin-induced cough reflex [95].

Several studies reported two new cysteine derivatives, S-carboxymethylcysteine-lysine salt [96, 97], N-acetylcysteine L-lysinate (Nacystelyn) [98], for water-soluble pharmaceutical forms. Thanks to the lysine, the compounds are better tolerated by the gastroenteric tract than other mucoactive drugs. This allows the administration of the drug at higher doses, resulting in more effectiveness of the drugs in clinic. We also are investigating the cysteine derivatives in our systems.

4. Future directions

Various parameters (chemical properties, physical properties, mucus production, surfactant phospholipids production, and mucociliary clearance) are considered to be important for the dynamics and the mobilization of airway secretions. Pharmacological investigation with appropriate techniques on the ability of an agent to modify these parameters can give us useful information about its mechanism of action. However, since these parameters are strictly interconnected, it is so complicated to understand the mechanism of action of mucoactive drugs. This means that the final aims, the reduction and the control of the obstructive symptoms, cannot always be achieved by the modification of a single parameter, but should more realistically be attributed to a general renormalization of several parameters. On the basis of this idea, it will be taken for granted that glucocorticoids are ideal mucoactive drugs, because glucocorticoids possess various pharmacological effects in the lung. From polypharmacological points of view, Chinese traditional medicines may belong to glucocorticoid-like drugs because Chinese medicines consist of many kinds of active components that have various pharmacological effects.

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