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Mucin gene expression and mucin secretion in human airway epithelium*

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INTRODUCTION

The amount of mucus produced in the human airway is mainly balanced by the rate of production and clearance through its resorption, vaporisation and ciliary transport. Many clinical problems occur when this balanced production and clearance of mucus is violated. Mucus (Figure 1) induces nasal obstruction and dyspnea by interrupting the air flow in the human airway and also aggravates coughing by promoting the adhesion of inspired noxious substances to airway mucosa. However, it also works as a physical barrier to many harmful materials by preventing infectious agents from attaching to the respiratory epithelium and by inactivating various toxic materials (Wanner, 1979; Kim et al., 1985).

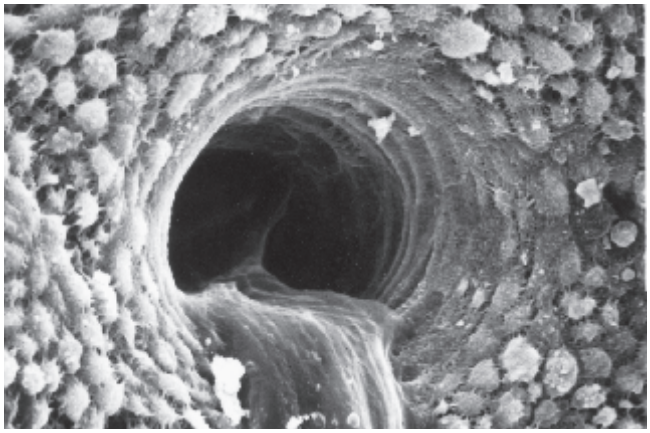

Figure 1. Scanning electron microscopy of human nasal inferior turbinate. Mucus coming out of the glandular duct is noted.

Two frequent pathologic changes observed in the respiratory epithelium are squamous metaplasia of the mucociliary epithelium and hypersecretion of mucus as a result of hyperplasia of the mucous secretory cells. A lot of research has focused on developing methods of restoring the epithelium from squamous metaplasia back to the mucociliary epithelium, and on seeking solutions to reduce mucus hypersecretion. The traditional strategies for controlling mucus hypersecretion include reducing the amount of mucus itself, enhancing the capability of the epithelium to transport mucus, and changing the rheological properties to establish a more effective coughing system for faster removal of mucus secretion. Among these three methods, reducing the amount of mucus secretion is the most effective therapy and also the most commonly employed method in clinical medicine. Many other research studies are currently in progress to control mucus hypersecretion. Advances in this area owe a lot to the introduction of molecular biologic techniques and the identification of mucin genes. However, further studies are still required to clarify the exact mechanism of regulation of mucus secretion in the human airway epithelium.

Hypersecretion is a common complication frequently observed in many respiratory diseases such as rhinitis, sinusitis, otitis media, nasal allergy, chronic bronchitis and cystic fibrosis. Various airway secretions are actively produced by the epithelium. Airway epithelium is mainly composed of two zones of epithelia, one covering the respiratory mucosa and the other covering invaginated area that forms the glandular structures. Two types of secretions are produced by airway epithelium, mucous secretions and non-mucin secretions. There are many kinds of non-mucin secretions such as lysozyme, lactoferrin, secretory Ig A, peroxidase, secretory leukocyte protease inhibitors (SLPI) and albumin (Basbaum et al., 1989, 1990). Mucins represent the major mucous secretion and may be as diverse as non-mucin secretions. Currently, secretory cells in the respiratory epithelium are classified as mucous or serous cells based on histological and biochemical studies. Generally it has been thought that mucous cells secrete mucin, and serous cells produce non-mucin secretions such as lysozyme, lactoferrin, albumin and SLPI (Sallenave et al., 1994). However, the mechanism that regulates the secretion of different secretory products is still not sufficiently understood. Furthermore, according to the latest reports, cytokines (inflammatory mediators), growth factors and extracellular matrix are also known to be involved in the regulation of cellular secretions (Guzman et al., 1995; Levine et al., 1995; Yoon et al., 1997). There is a lot of ongoing research on the clinical application of airway secretion. For example, this research includes many active studies on lysozyme, a natural anti-bacterial substance in the human body which could react with lipopolysaccharides (LPS) released by gram-negative bacteria. By unveiling the mechanism of lysozyme secretion and its nature in detail, it may be possible to prevent, or at least to reduce the rate of death due to the shock caused by LPS in AIDS or immunocompromised patients (Jolles et al., 1984; Takada et al., 1994). Moreover, SLPI, a substance that could prevent tissue damage caused by the proteolytic enzyme,

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is also under active research. Meanwhile, many cDNAs of mucin genes are being discovered. These discoveries have not only raised interest in the role of mucins but has opened a new chapter in the research of mucin and its secretion.

EPITHELIAL CELL CULTURE AND DIFFERENTIATION

There have been many studies aimed at exploring the biochemical functions, physiology and pathology of airway epithelial cells. Many laboratories have been focusing on developing various culture techniques for airway epithelial cells. These include: submerged technique - attaching epithelial cells on a plastic culture dish (Ostrowski et al., 1995); suspension technique - suspending epithelial cells in a culture media (Bridges et al., 1991); floating technique - first culturing epithelial cells on a collagen gel then floating the collagen gel along with the attached cells (Emerman et al., 1977; Yoon et al., 1995); and Air-Liquid Interface (ALI) technique - originally developed as a culture method for cornified epithelial cells in 1980 and now recently modified for the culture of airway epithelial cells (Adler et al., 1987). The ALI technique is especially unique in that cells are cultured on a semi-permeable porous membrane leaving the ciliated cell surface exposed to the air while the nutrient supports for cell growth are delivered only through the basal compartment of the cells similar to the normal physiological environment. This culture system has proven to be more effective for differentiating epithelial cells into secretory and ciliated epithelial cells. However, the method is not the only important factor in culturing epithelial cells. The supplements of the culture media also make significant differences in the direction of cellular differentiation. Therefore, it can be easily seen by reviewing the literature that supplements for culture media are very much different from one laboratory to another. It can also be seen that culture media can be largely classified into two groups, serum-free and serum containing media. The major reason for supplementing the culture media with serum is that it promotes the growth of airway epithelial cells, but as a consequence the analysis of the response of the epithelium to certain substances is obscured.

In other words, if a particular substance being examined shows no effect at all, it is very difficult to interpret the result due to the possibility that this particular substance is already found at a saturating concentration in serum. This is the reason why researchers in many laboratories around the world are trying to create a defined culture medium in which all the supplemental components added to a culture medium are known exactly, even though many problems still exist in the process of culturing the cells using truly defined culture media. Presently, bovine pituitary extract (BPE) is the preferred supplement to inhibit the growth of fibroblasts in culturing airway epithelial cells. Many investigators use BPE prepared in their laboratories with bovine pituitary glands purchased from Pel-Freez Co. (USA). Although the culture media containing BPE has numerous advantages over one with serum and is regarded as serum-free media in most articles, it still contains numerous undefined components.

To date, airway epithelial cell culture has been reported to be successful using guinea pig (Whitcutt et al., 1988; Adler et al., 1990), hamster (Niles et al., 1988; Moller et al., 1989), rat (Kaartinen et al., 1993; Davenport et al., 1996) and human (Wu et al., 1990; Hanamure et al., 1994), and cell lines possessing similar morphological and functional characteristics with human airway epithelium have been cultured. However, if the cells were grown submerged with media in plastic culture dishes, their capacity to differentiate into mucociliary epithelia has been noticeably diminished to a variable extent. On the other hand, a recent article has reported the presence of ciliated cells as well as mucus-secreting cells in cultures of submerged hamster airway epithelium (Lee et al, 1984), suggesting possible differences in the differentiation potential of different species.

Although the ideal would be to use human airway epithelia in each experiment we carry out, it is practically impossible to obtain enough cells from donors to perform the numerous groups of experiments (Gray et al., 1996). And, if primary epithelial cells are to be collected from multiple donors, there will be a problem of donor-to-donor variation in interpreting the results and also a high risk of contamination of collected specimens during the culture. In 1996, Dr. Nettesheim et al. of the National Institute of Environmental Health Sciences (NIEHS) succeeded in maintaining the characteristics of cultured cells in each passage of human tracheobronchial epithelial cells without any loss of potential capacity to differentiate into mucociliary epithelium. In these studies, the number of cells could be multiplied sufficiently to conduct a large number of experiments. The developed experimental models of retinoic acid-deficiency induced squamous metaplasia and mucus hypersecretion using the same epithelial culture system. Cultured cells from this system are being widely utilised by many different laboratories. Besides cultured human airway epithelial cells, SPOC1 (rat tracheal epithelial cell line), 9HTE$_o^-$: human tracheal epithelial cell line, and BEAS-2B: humanbronchial epithelial cell line are somewhat easier to work with and can be used for similar experiments.

MUCIN

Mucins are high molecular weight, polydisperse proteins that are heavily glycosylated. In fact, glycosylation accounts for about 80% of mucin's total dry weight. There are two types of mucin: secretory and membrane-bound (Rose, 1992). Secretory mucin is mainly produced in the respiratory, gastrointestinal and genitourinary tracts. The secretory mucin is related to the secretion of highly viscous mucin, and is made up of core peptides connected to many glycoconjugates forming huge oligomers. The mucous gel that forms from secreted mucin is responsible for mucociliary transport as well as protection of mucous membrane from dehydration or invasion by infectious organisms (Kim et al., 1991; Gum et al., 1992). Meanwhile, membrane-bound mucin does not form oligomers, but it can protect epithelial cells from being injured by inflammatory cells. It can also be secreted and is known to have certain roles in the differentiation of epithelial cells (Gendler et al., 1991; Parry et al., 1992; Aplin et al., 1994). Owing to the presence of many O-

linked glycan chains of core peptides, it has been difficult to disclose the amino acid sequence of mucin by traditional methods. Little is known about the specificity of O-glycosylation in airway epithelial cells. There are four major types of O-glycans. One of the major four glycan core types may be biosynthetically elongated at serine and/or threonine residues in tandem repeat domains; a different core structure may be elongated in threonine/serine/proline-rich domains; and a third core structure may be generated in cysteine-rich or other domains (Rose et al., 1997). Identification of the structure of O-glycans linked to each MUC apoprotein will be very important to investigate the role of specific mucins in many diseases. MUC proteins also have several N-glycosylation sites in amino and/or carboxyl termini domains.

Many research methods have been introduced for the study of mucin. First, AB-PAS histochemical staining (Figure 2) has been used to distinguish sialylated or sulfated glycoproteins from the neutral types. Second, lectin immunohistochemical staining (Figure 3) has been valuable for identifying types of glycoconjugates. Third, indirectly analysis of the amount of production and secretion of mucin has been performed by tagging radioisotopes ([^{3}H]glucosamine, [^{14}C]threonine, [^{35}S]sulfate) on the amino acids consumed for mucin production. However, this method has limitations in distinguishing proteoglycans such as hyaluronic acid, heparin sulfate, keratin sulfate and chondroitin sulfate, from mucin. Therefore, size-exclusion chromatography (Cheng et al., 1981), or the use of proteoglycan-degrading enzymes (Adler et al., 1987) before tagging the radioisotopes, has been applied for more specific mucin detection. Monoclonal antibodies for mucin were recently developed and have enabled a more quantitative immunoblot assay for the amount of mucin produced. Another advance is the recent progress in the laboratory of Dr. Sheehan toward identification of the major mucin of human trachea. However, considering the problem of verifying the efficacy of mucin antibodies that are often against glycosyl moieties, development of core peptide-specific antibodies has currently been of the utmost importance. Therefore, many efforts are being made to develop specific antibodies for each mucin gene to clarify the role and characteristics of mucin in many related diseases.

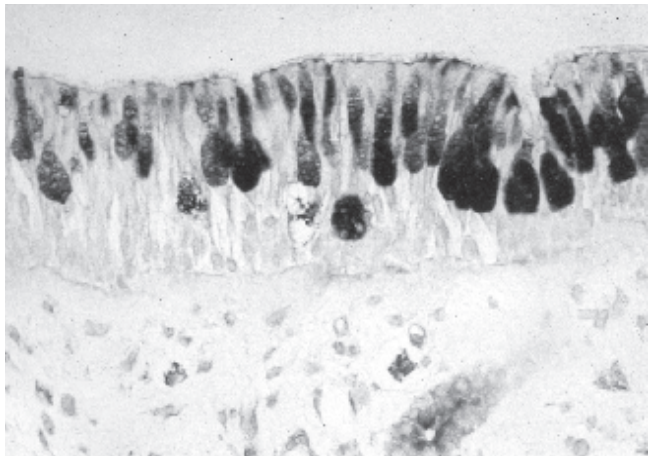

Figure 2. AB-PAS stain of human nasal inferior turbinate. Goblet cells were stained with AB or PAS.

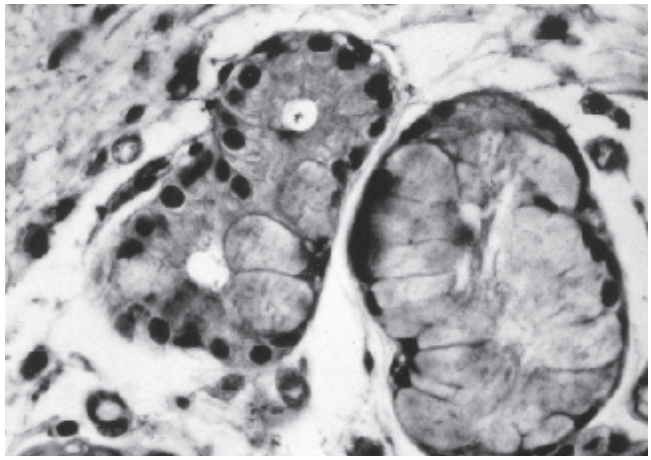

Figure 3. Lectin (Triticum vulgare, WGA) stain of submucosal glands. Only mucous cells were stained with WGA. The finding that there were mucous cells in serous acini suggested that serous cells might transdifferentiate into mucous cells under certain circumstances.

MUCIN GENES

Since there are many obstacles to overcome before unveiling the detailed structure and functions of mucin by biochemical and immunological methods, research on mucin is proceeding by analysing mucin genes (Rose and Gendler, 1977). Although the structure of the cDNAs of numerous mucin genes has been determined using molecular biological techniques, and many kinds of mucin genes have been detected in airway mucosa by in situ hybridisation (Audie et al., 1993; Aust et al., 1997), it sill is not clear which mucin gene has a major impact on mucin secretion. It is meaningful to notice that mucin mRNA is expressed before the actual mucin secretion, and the time lapse between the two events is thought to be caused by the time required for the formation of apomucin and glycosylation of the core peptide. To date, 9 different mucin genes, from MUCl to MUC8 (MUC5 is composed of two genes, MUC5AC & MUC5B), have been reported (Gerard et al., 1990; Gum et al., 1990; Aubert et al., 1991; Porchet et al., 1991; Hollingworth et al., 1992; Bobek et al., 1993; Toribara et al., 1993; Meerzaman et al., 1994; Guyonnet-Duperat et al., 1995; Gum Jr et al., 1997; Shankar et al., 1997; Toribara et al., 1997). MUCl is the only membrane-bound type and the rest are known to be the secretory type. Among the 9 mucin genes, 8 of them (MUCl, MUC2, MUC3, MUC4, MUC5AC, MUC5B, MUC7, MUC8) appeared to be expressed in human airway epithelium by Northern blotting and in situ hybridisation, but the exact role of each mucin gene is still unclear. Of these, the complete cDNA sequence is available for only three, MUCI, MUC2, and MUC7. Moreover, MUC2 is the only mucin gene with its entire genetic sequence clearly identified (Gum et al., 1997), and the genetic sequence of MUC5AC promoter is expected to be studied soon.

MUC1 is different from the other mucins in several aspects. MUC1 is a membrane- bound type rather than a secretory type because it has a transmembrane domain. The size of MUC 1 is smaller than that of other mucin genes. Heavy glycosylation is not seen in MUCl as in other types of mucin genes since there

are only 3 cysteine residues for each protein monomer. MUC1 maintains cellular polarity and stabilises cellular integrity, and is also involved in a step-by-step cellular recognition. In some circumstances, its expression increases with that of MUC5 (Guzman et al., 1996). MUCl is not expressed in the state of squamous metaplasia, implying that it is closely related with mucous differentiation of epithelial cells. Presently, MUCl is known to be expressed in the primary culture of human nasal and tracheobronchial epithelial cells (Gerard et al., 1990).

MUC2 is composed of more than 4,500 amino acids, and was first found in the small and large intestines, but, at present, it is also known to exist in the respiratory tract. However, An et al. (1994) reported that MUC2 may not be the major mucin gene in human respiratory mucosa by calculating the amino acid sequence inversely from the gene. Rats are the most frequently used animals for studies of respiratory diseases. In rat, MUC2 was not expressed in the surface epithelial cells, but was only expressed in the submucosal glandular tissues. Also, MUC2 expression was not observed in cultured airway epithelial cells of rodents (Guzman et al., 1996).

MUC3 (Aubert et al., l991; Gum Jr et al., 1997) gene encodes a very large glycoprotein with a structure very different from that of any mucin currently described and is not expressed in human tracheal and nasal mucosa. For MUC4 (Porchet et al., 1991), only the tandem repeat is presently known and research is lacking for more detailed information. MUC3 was isolated from the cDNA library of the human small intestine, but MUC4 and MUC5 were extracted from a tracheobronchial library.

MUC5 (Hollingworth et al., 1992; Bobek et al., 1993; Toribara et al., 1993) is a gene for which a partial peptide sequence has been found in airway mucin. Most research on respiratory mucins has focused on MUC2 and MUC5. MUC5A, 5B and 5C localised to chromosome 11pl5 and thus were originally proposed to be on the same gene.

However, further analyses demonstrated that cDNA clones encoding MUC5A and MUC5C, but not MUC5B, were on the same gene. Nevertheless, MUC5-specific antibodies are presently not available, and there is no way to prove whether MUC5 is a major contributor to the mucin produced in human airway epithelium. Thornton et al. (1996) reported that MUC5AC-induced mucin is the major mucin in normal human airway epithelium, but not in patients with chronic bronchitis. Hovenberg et al. (1996) reported that MUC5AC mucin from human trachea originates from goblet cells and this glycoprotein is not a major product of the submucosal glands. Thornton et al. (1997) reported that MUC5B is one of the major mucins in the respiratory tract and there are two mucin populations which are products of the MUC5B gene, but they represent different glycoforms.

These findings suggest that MUC5AC and MUC5B mucin are major in human trachea. MUC5B appeared to be one of the predominant submucosal gland mucins in the turbinates (Aust et al., 1997). Presently, active research is also in progress about other mucin genes; MUC6 (Meerzaman et al., 1994; Torbara et al., 1997) in stomach, MUC7 (Guyonett-Duperat et al., 1995) in submandibular gland. MUC8 (Shankar et al., 1997) gene maps to chromosome 12q24.3 and is known to be expressed in both goblet cells and submucosal glands in human tracheal epithelium.

DETECTION METHOD OF MUCIN GENE mRNA

The mRNAs of most secretory mucin genes found in humans are polydisperse and are not easily detected with traditional methods such as Northern blotting due to the presence of a smear, but quantitative analysis of various mucin is essential for understanding the pathophysiology of many diseases associated with mucus hypersecretion. Several methods have been developed to overcome this limitation: a dot blot assay (Voynow et al., 1994), a competitive RT-PCR using an internal control (Guzman et al., 1996) or Ribonuclease Protection Assay (Masuda et al., 1996). The advantages of competitive RT-PCR over the dot-blot assay are that there is no need for radioisotope tagging, it is less time-consuming, more sensitive and capable of reducing the possibility of background caused by non-specific signals since the actual size of PCR products can be measured. The specificity of the RT-PCR assay can vary. For instance, some researchers insist on using MIMIC RNA instead of MIMIC DNA for its internal control. Meanwhile, some sceptical researchers with doubts about the results of RT-PCR stress the need to reconfirm the results of RT-PCR with traditional detection methods owing to the fact that RNA collected under different conditions may show different reverse transcription efficiency and, even under the same conditions, the reverse transcription efficiency can still be different.

They further insist that the reverse transcription efficiency can vary not only among different RNAs, but even in designing primers for the same gene according to the site of the primer design. Nevertheless, in situations where there is no better alternative, researchers favouring competitive RT-PCR urge that the claims of researchers against RT-PCR are not sufficiently persuasive. Competitive RT-PCR for the quantitative detection of mucin gene mRNA was first introduced by Guzman et al.(1996) and RPA is being commonly used in some laboratories (Masuda et al., 1996). Recently, Debailleul et al. (1998) reported that mucin gene transcripts can be detected as distinct bands by Northern blot analysis using an improved method to isolate intact large RNA.

MUCOUS DIFFERENTIATION AND SQUAMOUS METAPLASIA OF EPITHELIAL CELLS

In general, knowledge about factors that induce mucous differentiation is very limited due to the lack of research on the process of mucous differentiation of epithelial cells. Nevertheless, research on epidermal growth factor (EGF) and retinoic acid (RA) are relatively well advanced. EGF has been reported to promote differentiation of secretory cells in tracheobronchial mucosa (St. George et al., 1991), which is contrary to the results of our study indicating that the effect of EGF on mucous differentiation is very limited (unpublished data). Meanwhile, RA is the essential factor in most epithelial cell cultures and, according to our results, the concentration of RA should be maintained at more than 10^{-9}M for epithelial cells to differentiate into mucociliary epithelium.

If the concentration is less than 10^{-9}M, epithelial cells invariably differentiate into squamous epithelium regardless of the concentration of EGF, hence, RA is considered as an essential factor in mucociliary differentiation. Koo et al., (l998) depleted RA from the culture media and intentionally induced squamous metaplasia, then supplemented RA at the early and late stages of squamous metaplasia to see if the squamous epithelium was capable of being differentiated back into mucociliary epithelium. All of the squamous epithelia were recovered and differentiated back into mucociliary epithelia regardless of the degree of squamous metaplasia. Therefore, squamous metaplasia in airway mucosa is thought to be induced by local RA depletion caused by secondary effects of inflammatory mediators and chronic irritations.

MATERIALS INDUCING MUCUS SECRETION AND ITS MECHANISM

Mucin secretion of airway epithelial cells is affected mainly by irritating gases or inflammatory mediators. In other words, mucin secretion is known to increase when the cells are exposed to ozone, endotoxin, SO_2, virus or inflammatory mediators such as IL-4, tumor necrosis factor (TNF)-α and neutrophil elastase (Harkema et al., 1989, 1991; Jany et al., 1991; Young et al., 1996). Nowadays, much effort is being focused on revealing the mechanism for regulating mucin secretions. Among the inflammatory mediators, eicosanoids such as Platelet Activating Factor (PAF) and $PGF_{2\alpha}$ are regarded as the major stimulators of mucin secretion.

According to a recent report, mucin secretion is increased by the interaction of adenosine triphosphate (ATP) with P_2 purinoreceptors on the cell membrane to which phospholipase C (PLC) is connected (Kim et al., 1991; Davies et al., 1992; Kim et al., 1993). Larivee et al. (l994) reported that PAF increases mucin secretions and that it can be completely blocked by inhibitors of protein kinase C (PKC). According to the report of Kai et al. (1994), 4b-phorbol 12a-myristate 13-acetate (PMA) can upregulate the mucin secretions in a hamster' s tracheal epithelium, which was also sufficiently blocked by PKC inhibitors. Meanwhile, Ko et al. (1997) described that mucin secretion induced by ATP could not be completely inhibited when desensitised with PKC after pretreatment with PMA. This implies that there might be some other signal transduction pathway for mucin secretion induced by ATP other than PKC. Wright et al. (1995) also reported that Reactive Nitrogen Species (RNS) is responsible for the increase of mucin secretion through the signal transduction pathway of PLC, but predicted it was not associated with PKC. Li et al. (1997) presented in their study results that LPS induced hypersecretion of mucin, and that it was completely blocked by genistein or tyrphostin AG126, which are tyrosine kinase inhibitors.

Thus, septic shock, a major cause of death in cystic fibrosis, might be avoidable with LPS antagonists or tyrosine kinase inhibitors, and eventually the morbidity and mortality of cystic fibrosis may be expected to decrease.

CONCLUSION

Many efforts are being made to analyze the characteristics of secreted mucin in diverse airway diseases, to clarify the mechanisms of mucin secretion and eventually to regulate various mucin genes. At present, cloning of the promoter for MUC2 has been completed, and cloning of the promoter for MUC5AC is expected to be completed soon. Also, active research is in progress to determine the definite signal transduction pathways (PKC, arachidonic acid cascade, NOS and MAP kinase) related with mucin secretions, as well as the efficacy of inhibitors blocking those pathways.

Moreover, since a new technique has been tried to inhibit the transcription of mucin genes by performing transcriptional activation assays with mucin gene promoters, it is anticipated that a new type of medication, more specific than any other drugs used to inhibit mucin secretion in the past, will be developed soon.

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