Platelet-derived growth factor (PDGF) induces *MUC8* gene expression in human airway epithelial cells

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My wife, Eun Sung Kang, has assisted me in innumerable ways. I dedicate this thesis to her with genuine affection. I would like to share my pleasure with lovely daughter Won-ji and cute son Seong-hyeon.

Finally, I hope that I will do an in-depth study for the next years without indolence and conceit, and that every person around me will encourage me doing so.

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ABSTRACT

Platelet-derived growth factor (PDGF) induces *MUC8* gene expression in human airway epithelial cells

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Reactive oxygen species (ROS) which are either directly inhaled or generated by cytokines or growth factors, cause inflammation in the airway epithelial cells, increase secretion of mucus, and lead to chronic inflammatory airway disease. Hydrogen peroxide (H_2O_2), which is a common type of ROS, is formed by various pathways in the intracellular system. Platelet-derived growth factor (PDGF) is known to increase the generation of hydrogen peroxide in a few non-phagocytic cells. We intended to verify that there was a change in the expression of mucin gene induced by PDGF in human airway epithelial cells, and that H_2O_2 induced by PDGF caused an increase in the expression of mucin gene. RT-PCR results showed that expression of MUC8 mRNA was increased in PDGF-treated NCI-H292 cells. By applying chemical probe 2′7′-Dichlorofluorescin diacetate (DCFH-DA) to the cells, and using flow cytometry to quantify fluorescence, we found that the production of intracellular hydrogen peroxide increased in PDGF-treated NCI-H292 cells. However, when the cell was pre-treated

with the antioxidant N-acetylcysteine (NAC), neither increase in the formation of intracellular H_2O_2 nor over-expression of *MUC8* mRNA were observed. In this study, we confirmed that intracellular H_2O_2 is produced by PDGF in human airway epithelial cells, and also that, among various mucin genes, the expression of *MUC8* gene increases in consequence.

Key Words : reactive oxygen species (ROS), *MUC8*, platelet-derived growth factor, hydrogen peroxide, human airway epithelial cells.

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I. INTRODUCTION

Mucin hypersecretion is a major pathologic manifestation of chronic inflammatory airway diseases such as rhinitis, sinusitis, otitis media, nasal allergy, chronic bronchitis and cystic fibrosis. Mucin is a major component of the mucus produced by epithelium of the respiratory tracts.[1-3] Currently 20 different mucin genes have been identified and these are subdivided into three groups; membrane-associated mucins, secreted gel-forming mucins, and secreted non gel forming mucins. *MUC1, MUC3, MUC4, MUC11, MUC12, MUC 13, MUC17, MUC18, MUC20* are generally known as membrane-associated mucins, while *MUC2, MUC5AC, MUC5B, MUC6* are generally known as secreted gel-forming mucins. On the other hand, *MUC7, MUC8, MUC9* are considered as secreted, non gel-forming mucins.[4-6] Expression of *MUC8* is generally recognized to be increased in pathologic conditions.[7]

The oxidative injury triggered by both inhaled and locally generated reactive

oxygen species (ROS) elicit an inflammatory response that can profoundly impair the structural integrity and biological properties of bronchial epithelium. There are a large number of studies providing that ROS, such as hydrogen peroxide (H_2O_2), superoxide anion (O_2^-) and hydroxyl radical (OH・), play a critical role in the initiation and progression of a great diversity of airway diseases.[8]

Hypersecretion diseases of airways are associated with abnormal cell growth and differentiation in mucus-synthesizing cells including submucosal hypertrophy and goblet cell hyperplasia. Mucin synthesis in airways is regulated by growth factor receptor system. The regulation of gene expression by oxidative stress involves numerous signaling pathways including mitogen-activated protein kinase(MAPK) triggered by receptor tyrosine kinase such as platelet-derived growth factor receptor (PDGFR) and epidermal growth factor receptor (EGFR). Protein tyrosine kinases are essential for the activation and proliferation of inflammatory cells and airway-resident cells. Thus, chronic exposures to low levels of ROS may cause abnormal cell growth and differentiation.[9]

Platelet-derived growth factor (PDGF) is a major mitogen for fibroblast, smooth muscle cells and other cells. Structure of PDGF is a family of heterodimeric or homodimeric isoforms of A- and B- polypeptide chains that are synthesized as precursor molecules undergoing proteolytic maturation.[10] PDGF has shown to lead to hyperplasia of airway smooth muscle cells, epithelial cells, and goblet cells.[11] Overactivity of PDGF has been associated with chronic airway diseases, including asthma and fibrotic pulmonary conditions. Alveolar macrophages from patients with idiopathic pulmonary fibrosis exhibited enhanced expression of PDGF-BB compared to macrophages from normal individuals.[12] Using transgenic mice, it was demonstrated

that control of the lung surfactant is regulated by PDGF-BB expression; these mice developed several pulmonary abnormalities, including emphysema and fibrosis.[13]

We hypothesized that hydrogen peroxide, the endogenous production of which is induced by PDGF, increases mucin gene expression in human airway epithelial cells.

In this study, we examined whether PDGF produced intracellular H_2O_2 production in human airway epithelial cells and investigated the effect of PDGF on the expression of mucin genes in human airway epithelial cells.

II. MATERIALS AND METHODS

1. Reagents

Platelet-derived growth factor (PDGF-BB) was purchased from Calbiochem (San Diego, CA, USA). N-acetyl-cysteine (NAC) was purchased from Sigma Aldrich (St. Louis, MO, USA). 2′7′-Dichlorofluorescin diacetate (DCFH-DA) was purchased from Molecular Probes (Eugene, OR, USA). DCFH-DA was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mM.

2. Cell cultures

The human lung mucoepidermoid carcinoma cell line (NCI-H292) was purchased from American Type Culture Collection (CRL-1848, Manassas, VA, USA) and cultured in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum in the presence of penicillin/streptomycin at 37℃ in a humidified chamber with 5% CO_2. For serum deprivation, confluent cells were washed twice with phosphate-buffered saline and recultured in RPMI 1640 with 0.2% fetal bovine serum.

3. Experimental conditions

PDGF was diluted in HCl to stock concentrations of 50 μM. The PDGF stock was further diluted in RPMI to experimental concentrations of 1, 5, 10 and 100 ng/ml. For RT PCR, NCI-H292 cells were treated with PDGF(1, 5, 10, 100 ng/ml) for 24 h prior to evaluation of *MUC1, MUC4, MUC5AC, MUC7* and *MUC8* gene expression. To test the effect of NAC on gene expression, cells were pre-treated with NAC (30 mM) for 1 h and then they were incubated in fresh medium containing 5 ng/ml PDGF for 24 h.

4. Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from NCI-H292 cells treated with PDGF (1, 5, 10, 100 ng/ml) using Trizol (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized with random hexamer primers (PerkinElmer Life Sciences and Roche Applied Science, Boston, MA, USA) using Molony murine leukemia virus-reverse transcriptase (PerkinElmer Life Sciences and Roche Applied Science, Boston, MA, USA). Oligonucleotide primers for RT-PCR were designed according to published sequence of *MUC1*, *MUC4*, *MUC5AC*, *MUC7* and *MUC8* (Table 1)[6,7,23].

Table 1. The experimental conditions of RT-PCR and sequences of oligonucleotides used in RT-PCR

Product	Cycle	Annealing Temp(℃)	Primer	Sequence of oligonucleotide
MUC1	35	55	Forward Reverse	CTC ACC TCC TCC AAT CAC GAA TGG CAC ATC ACT CAC
MUC4	30	58	Forward Reverse	TGG AAC CAT TTC TGC AAT CA GAG GAA GGC CAT GTT GTT GT
MUC5AC	35	60	Forward Reverse	CCA CAA CTA CTT CTG CGG TGC GCA CTC ATC CTT CCT CAT GTC
MUC7	35	55	Forward Reverse	CCA CAC CTA ATT CTT CCC CTA TTG CTC CAC CAT GTC
MUC8	35	55	Forward Reverse	ACA GGG TTT CTC CTC ATT G CGT TTA TTC CAG CAC TGT TC
β2-M	23	55	Forward Reverse	CTCGCCCTACTCTCTCTTTCTGG GCTTACATGTCTCGATCCCACTTAA

* β2-M : β2-microglobulin

RT-PCR products were resolved in a 2% agarose gel (FMC, Rockland, ME, USA) and visualized with ethidium bromide under a transilluminator. To confirm that the amplified products were from mRNA and not from genomic DNA contamination, negative controls were performed, omitting the reverse transcriptase and no PCR products were observed.

5. Measurement of intracellular H_2O_2

Amount of H_2O_2 generation was measured using an oxidation-sensitive fluorescent probe, 2'7'-dichlorofluorescin diacetate (DCFH-DA). The oxidized form (2'7'-dichlrofluorecin, DCF) is highly fluorescent. Two experimental methods were used for the measurement of DCF fluorescence: confocal microscope and flow cytometry.

Confocal microscope: NCI-H292 cells were first cultured for 3 days and then treated with PDGF for 10, 20, 30 min. The cells were washed with HBSS medium without phenol red and incubated in the dark for 10 min in HBSS solution containing 10 uM DCFH-DA. Culture dishes were transferred to a Zeiss Axiovert 135 inverted microscope, equipped with a X20 Neofluor objective and Zeiss LSM 410 confocal attachment, and ROS generation was detected as a result of the oxidation of DCF (excitation; 488 nm, emission; 515-540 nm). The effects of DCF photo-oxidation were minimized by collecting the fluorescent image with a single rapid scan (line average; 4, total scan time; 4.33 s) and using identical parameters, such as contrast and brightness, for all samples. Five groups of 20-30 cells each were randomly selected from the image in the digital interference contrast (DIC) channel for each sample. Fluorescence intensity was measured for each group from the fluorescence image, and the relative fluorescence intensity was taken as the average of the eight values. Therefore, the

relative fluorescence intensity (given in arbitrary units) reflects measurements performed on a minimum of 100 cells for each sample.[14]

Flow cytometry : NCI-H292 cells were first cultured for 3 days and then treatment with PDGF for 10, 20, 30 min. The cells were washed with HBSS medium without phenol red. DCFH-DA (10 μM) was added for 15 min at 37℃ in the dark. The cells were washed with HBSS. Cells were detached by 250 mM Trypsin / 4 mM EDTA, and re-supended in PBS. Immediately after re-suspension, the formation of DCF were analyzed by Fluorescence Activated Cell Sorting (FACS) Calibur™ flow cytometer (Becton-Dickinson, Mountain View, CA, USA). The green fluorescence produced by DCF was measured by the FL-1 detector of the flow-cytometer. A-488 argon laser beam was used for excitation. The data were analyzed in WinMDI software.

6. Statistical analysis

Data are presented as the mean ± SEM of triplicate cultures from the same experiment. Statistical comparison was performed using Student's t-test. $p<0.05$ was considered statistically significant.

III. RESULTS

PDGF induced gene expression of MUC8, but not MUC1, MUC4, MUC7, or MUC5AC, in a dose-dependent manner.

To determine whether PDGF could induce the expression of mucin genes, RT-PCR was performed after treatment of NCI-H292 cells with PDGF (1, 5, 10, 100 ng/ml) for 24 h. Levels of *MUC8* mRNA increased after treatment with PDGF in a dose-dependent manner, while levels of *MUC1*, *MUC4*, *MUC7* and *MUC5AC* did not. *MUC8* gene expression was increased significantly at 5 ng/ml PDGF. No corresponding change was found in the expression of the internal control, β2-microglobulin (Fig. 1A).

PDGF induced gene expression of MUC8 in a time-dependent manner.

RT-PCR was performed after treatment of cells with PDGF (5 ng/ml) for 2, 6, 12, 24 h. Levels of *MUC8* mRNA increased in a time-dependent manner. *MUC8* gene expression was increased the most at 24 h. No corresponding change was found in the expression of the internal control, β2-microglobulin (Fig. 1B).

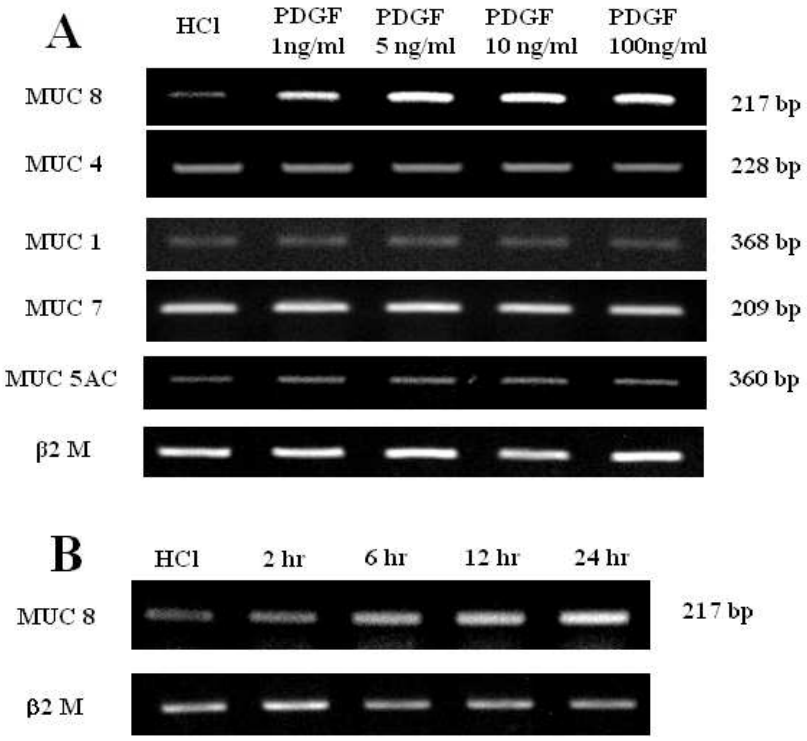

Figure 1. PDGF induced *MUC8* gene expression in NCI-H292 cells.

Confluent cells were treated with PDGF (1, 5, 10, 100 ng/ml) for 24 h. β2-microglobulin was used as an internal control. Levels of *MUC8* mRNA increased after treatment with PDGF in a dose-dependent manner, but did not affect expression of *MUC1*, *MUC4*, *MUC7* and *MUC5AC*. *MUC8* gene expression was increased significantly at 5 ng/ml PDGF (A). Confluent cells were treated with PDGF (5 ng/ml) for 2, 6, 12, 24 h. Levels of *MUC8* mRNA increased in a time-dependent manner. *MUC8* gene expression was increased the most at 24 h. β2-microglobulin was used as an internal control (B). All experiments were conducted at least three separate times.

PDGF increased intracellular H_2O_2 production in NCI-H292 cells.

We evaluated whether PDGF could induce production of H_2O_2 in human airway epithelial cells. NCI-H292 cells were incubated with H_2O_2-sensitive fluorophore DCF and were imaged by laser confocal microscopy before and after treatment with PDGF (5 ng/ml) for 10, 20, 30 min. Microfluometric study with confocal microscopy showed that, compared with quiescent cells, stimulation with PDGF (5 ng/ml) increased DCF fluorescence by 3 fold at 10 min. The PDGF-stimulated increase in H_2O_2 is transient, with H_2O_2 peaking with the first 10 minutes after PDGF addition and then returning rapidly toward basal levels (Fig. 2A).

Another method to detect H_2O_2 was performed using DCFH-DA and flow cytometry. An untreated control was run as a baseline to account for the presence of endogenous ROS. DCF fluorescence increased the most following the addition of PDGF (5 ng/ml) at 10 min. A histogram showed the right shift in DCF signal at 10 min. There is no right shift in DCF signal at 20, 30 min (Fig. 2B).

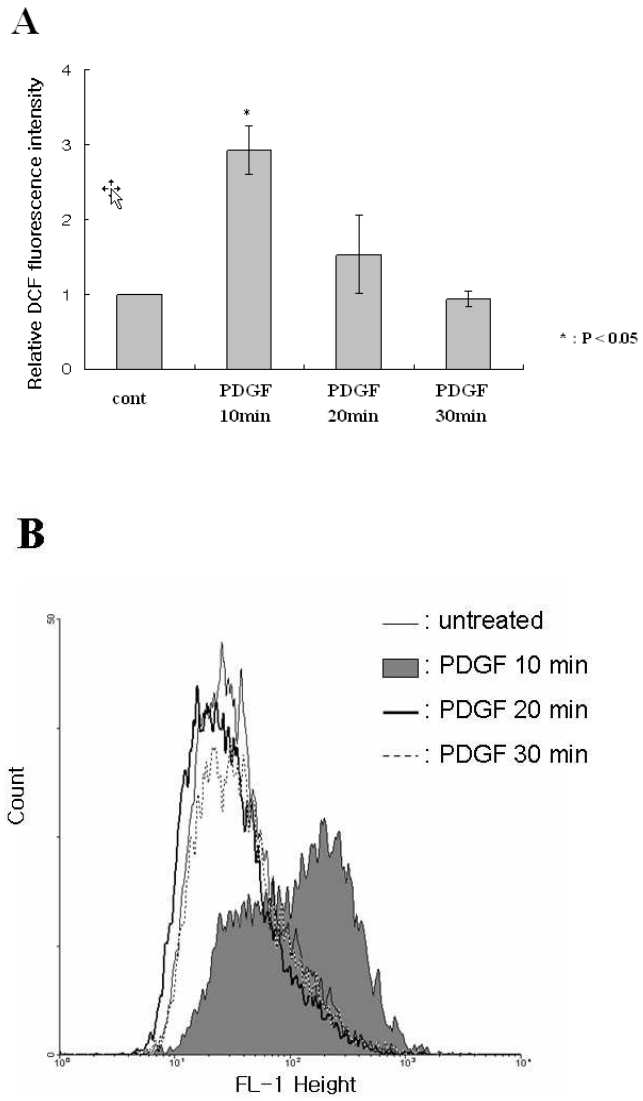

Figure 2. Production of H_2O_2 in NCI-H292 cells stimulated with PDGF.

NCI-H292 cells were incubated with H_2O_2-sensitive fluorophore DCF and were imaged by laser confocal microscopy before and after Treatment with PDGF (5 ng/ml) for 10, 20, 30 min. Microfluometric study with confocal microscopy showed that, compared with quiescent cells, stimulation with PDGF (5 ng/ml) increased DCF

fluorescence by 3 fold at 10 min. H_2O_2 generation after PDGF stimulation was measured in relative DCF fluorescence intensity at 10 min interval (Scales of 0 to 4 units). Values were expressed as mean ± SEM (A). Histogram showed that there is a right shift of DCF fluorescence signal at 10 min following the treatment of PDGF. There is no right shift of DCF fluorescence signal at 20, 30 min (B).

N-acetylcysteine (NAC) suppressed PDGF-induced H_2O_2 Production

We tested whether a reactive oxygen scavenger would have an inhibitory effect on PDGF-induced H_2O_2 production. When NCI-H292 cells were pre-treated with chemical antioxidant N-acetylcysteine (30 mM), a reduction in PDGF-stimulated DCF fluorescence was noted on confocal microscope (Fig. 3A).

Reduction of DCF fluorescence showed a left shift on the histogram when pre-treated with NAC, and this was confirmed by flow cytometry (Fig. 3B).

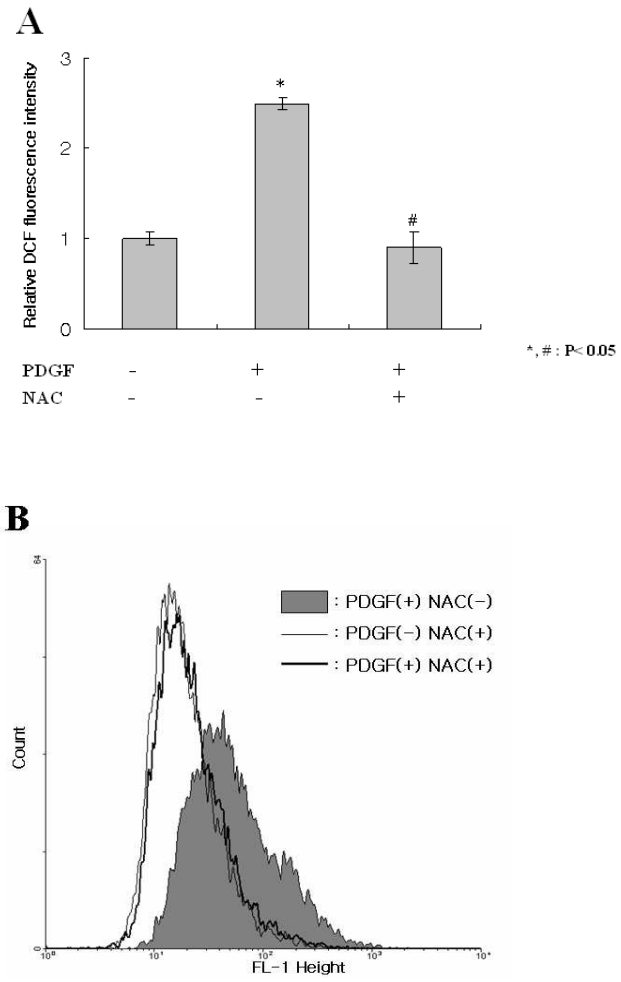

Figure 3. Effects of N-Acetylcysteine on PDGF-induced H_2O_2 production.

DCF florescence of cells either stimulated with PDGF or basal were used to identify a function of NAC. When NCI-H292 cells were pre-treated with chemical antioxidant N-acetylcysteine (30 mM), a reduction in PDGF-stimulated DCF fluorescence was noted. Values were expressed as mean ± SEM (A). Intracellular H_2O_2 assay was assessed by means of DCFH-DA on flow cytometry. The pre-treatment of NAC normalized the fluorescence in cells incubated with PDGF (B).

NAC suppressed PDGF-induced MUC8 gene expression.

We next evaluated whether NAC could suppress the gene expression of *MUC8*. Confluent cells were pre-treated with NAC (30 mM) for 1 h, then treated with PDGF (5 ng/ml) for 24h. RT-PCR analysis showed that treatment with PDGF increased *MUC8* gene expression compared with the control in NCI-H292 cells. Pre-treatment with NAC for 1 h followed by 24 h treatment with PDGF suppressed *MUC8* gene expression, compared to those treated with PDGF alone (Fig. 4).

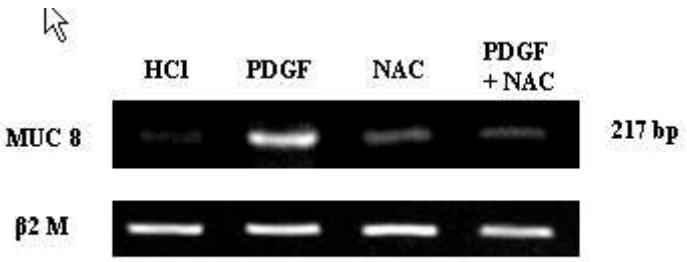

Figure 4. N-Acetylcysteine suppressed PDGF-induced *MUC8* gene expression.

Confluent cells were pre-treated with NAC (30 mM) for 1 h, then treated with PDGF (5 ng/ml) for 24 h. β2-microglobulin was used as an internal control. NAC suppressed PDGF-induced *MUC8* gene expression. This result showed that NAC inhibit PDGF-induced *MUC8* gene over-expression.

IV. DISCUSSION

Hypersecretion of mucus is the most common pathologic feature of various inflammatory airway diseases. Accordingly, understanding the expression and regulation of the mucin genes expressed in the airway is quite important. The airway epithelium is continuously exposed to both exogenous oxidants, including air pollutants and cigarette smoke, and endogenous oxidants generated by activated inflammatory cells through mitochondrial electron transport.[15]

ROS is classically thought of as cytotoxic and mutagenic or as inducers of oxidative stress, and recent evidence suggests that ROS play a role in stimulation or inhibition of cell proliferation, apoptosis, and inflammation.[16, 17]

In the present study, we showed that PDGF stimulated a transient intracellular H_2O_2 generation in human airway epithelial cells. This phenomenon agrees with the findings from other cell types, in which H_2O_2 was considered as the activator for redox signaling to promote certain cellular functions.[18-20]

We demonstrated that PDGF induced *MUC8* mRNA expression in human airway epithelial cells. PDGF did not have any effect on *MUC 1*, *MUC 4*, *MUC 7* or *MUC 5AC* mRNA expression. Previous studies have shown that *MUC5AC* mRNA expression was increased when treated with H_2O_2 in human airway epithelial cells.[21]

MUC8 may be one of the major mucin genes because inflammatory mediators upregulate *MUC8* gene expression in vitro.[22, 23] In addition, the *MUC8* gene expression level is increased in human nasal polyp epithelium when it is stimulated by various inflammatory mediators.[1] However, as only partial sequence (323 amino acids) of *MUC8* cDNA has been identified, its role in human airway epithelium remains unclear. The cDNA of *MUC8* gene and its promoter sequence have not been fully identified,

but when this is achieved, the function and regulation of *MUC8* in the human airway can be clearly explored.[7]

In previous studies, H_2O_2 was directly applied to the cell externally to investigate any changes in mucin gene expression. In these cases, H_2O_2 could act by itself by permeating the cell membrane, or create secondary ROS by binding to receptors on the cell membrane. Thus, seeing a need for a physiological model, we decided to measure intracellular H_2O_2 produced after treatment with a growth factor.

PDGF was known to produce intracellular H_2O_2 when treating it on phagocyte and non phagocyte externally, but there was no published study on human airway epithelial cells. In this study, it was confirmed that PDGF increases intracellular H_2O_2 in human airway epithelial cells and that the induced H_2O_2 acts as an oxidative stress which in turn, leads to increased expression of *MUC8* gene.

The role of H_2O_2 is known as killing cells by oxidative stress, however recent studies have revealed that H_2O_2 has its potential role in host defense aiding in the killing of ingested microorganism, oxidative biosynthesis such as Tyr cross-linking mediated by peroxidases, and signaling such as in mediating mitogenic signaling pathways.[24]

PDGF binding to receptors in many cell types is known to induce several signal pathways that lead to various cellular functions. It becomes clear in recent years that mitogenic action of PDGF is mediated by ROS generated during the process for the down stream signaling transduction cascade.[10] It was reported that PDGF-stimulated ERK1/2 activation induced arachidonic acid release to facilitate ROS production from NADPH oxidase in human lens epithelial cells.[18, 25]

Further studies on the signal pathways which lead to mucin gene expression when

stimulated by PDGF in human airway epithelial cells are required.

V. CONCLUSION

In summary, oxidative stress including H_2O_2 is a central feature in airway inflammatory diseases. We demonstrated that PDGF stimulated intracellular H_2O_2 production in human airway epithelial cells, and consequently, PDGF-induced H_2O_2 increased *MUC8* mRNA expression in human airway epithelial cells.

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induced signaling in lens epithelial cells. Mol Vis 2006;12:821-31.

ABSTRACT (in Korean)

사람 기도 상피에서 혈소판 유래 성장인자에 의해 유도된 *MUC8* gene 의 발현 증가

(지도교수 : 윤 주 헌)

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직접적인 흡입 또는 여러 성장인자나 활성 세포물질에 의해 체내에서 발생하는 활성산소는 기도에 염증을 일으키고, 점액 분비를 증가시키며, 만성 기도 염증 질환을 일으킨다. 이러한 활성산소 중 대표적인 것으로 과산화수소가 있으며 이는 세포내에서 다양한 경로를 통해 생성된다. 혈소판 유래 성장인자는 세포내에서 과산화수소의 생성을 증가시킨다는 것이 알려져 있다. 본 연구자는 사람 기도상피세포에서 혈소판 유래 성장인자에 의해 내인적인 과산화수소의 생성 증가 여부와 이로 인한 mucin gene 의 발현에 변화가 있는지를 확인해 보고자 하였다. 사람기도 상피세포에 혈소판 유래 성장인자를 처리시에 *MUC8* gene의 mRNA 발현이 증가하는 것을 역전사 중합 효소 반응을 통해 측정하였다. 사람기도 상피내에 혈소판 유래 성장인자 처리시에 세포내 과산화수소의 생성이 증가한다는 것을 2′7′-Dichlorofluorescin diacetate 의 형광 발현이 증가하는 것을 통해 확인할 수 있었다. 항산화물질인 N-acetylcysteine 을 전처치 할 경우, 혈소판 유래성장 인자를 처리시에

세포내 과산화수소 생성이 증가하지 않았고, *MUC8* gene의 mRNA 과발현도 나타나지 않음을 확인하였다. 본 연구에서 사람 기도 상피세포에서 혈소판 유래 성장인자에 의해 여러 mucin gene 중에 *MUC8* gene의 발현이 증가하는 것을 확인하였다.

핵심되는 말: 활성산소, *MUC8*, 혈소판 유래 성장인자, 과산화수소, 사람 기도 상피 세포