# Effects of steroids on mucins of human corneal epithelial cells

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# Effects of steroids on mucins of human corneal epithelial cells

Directed by Professor Jong Bok Lee

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A Dissertation for the the Degree of Dr. of Philosophy
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**Abstract**

Effects of steroids on mucins of human corneal epithelial cells

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The mucin gel layer is believed to comprise a major fraction of the tear film. Although the composition of mucins in ocular surface has been revealed, only minor progress has been made in the field of influences of steroid hormones, which comprise some of the most commonly used drugs in ophthalmologic practice, on the expression of mucins in ocular surface.

To examine whether mucin gene expression is influenced by steroid hormones in cultured human corneal epithelial cells (HCECs), the effects of estrogen and dexamethasone on the expression of MUC1 and MUC4, two known mucins produced by corneal epithelial cells were determined. HCECs were cultured in medium supplemented with estrogen or dexamethasone. The modulation of MUC1 and MUC4 expression by estrogen and dexamethasone were investigated by RT-PCR and Western blot analysis.

The expression of MUC4 mRNA and its protein were enhanced in HCECs by estrogen. Treatment of HCECs with dexamethasone caused a decrease in MUC4 mRNA and its protein. The effects of steroid hormones were abolished by both glucocorticoid antagonist (RU38486) and estrogen antagonist (tamoxifen). Steroid hormones appear to be implicated in the expression of mucin in HCECs. This study supports this role of steroids and furthermore suggests that

steroid receptors may be implicated in the induction of its expression. Thus, steroids may be important modulators of mucin in corneal epithelial cells.

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Key Words: estrogen, dexamethasone, MUC1, MUC4, corneal epithelial cell.

# Effects of steroids on mucins of human corneal epithelial cells

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The Graduate School of Yonsei University

Kyoung Yul Seo

## I. Introduction

The surface of the eye is overlaid by a tear film, which covers and protects the corneal and conjunctival epithelia. The tear film is biochemically complex, being composed of three layers: (1) an outer lipid layer, secreted by meibomian glands; (2) a middle aqueous layer, secreted mainly by lacrimal glands; and (3) an inner mucin-containing gel layer, produced by and associated closely with apical cells of the ocular surface. The mucin gel layer is believed to comprise a major determining factor of tear film thickness.[1-3]

The complex constitution and interactions of the tear film assure optical clarity, lubricate the ocular surface, and protect against pathogenic and noxious agents. In the ocular epithelium, secreted mucins appear to play an important role in the maintenance of the ocular surface and tear film and in the protection against external pathogens and foreign debris. [4-5]

The mucins are large glycoconjugates with molecular masses ranging from $3\times 10^5$ to over $4\times 10^7$ kDa. It has been difficult to characterize mucins either by biochemical or immunologic methods. The recent isolation and characterization of several genes encoding mucin has permitted the analysis of the molecular mechanisms involved in gene regulation. In addition, it has been demonstrated that regulation of mucin production is influenced by steroid hormones in many tumor and non-tumor cells. [6-11]

Several reports indicate that both corneal and conjunctival epithelia produce mucins.[12-14] These include the secretory mucins, MUC2, MUC4 and MUC5AC,[15, 16] and a membrane mucin, MUC1 by the conjunctival epithelium and MUC4 and MUC1 by the corneal epithelium.[17, 18] Although the composition of mucins on the ocular surface has been revealed, only minor progress has been made in elucidating the influences of steroid hormones on their expression of mucins in ocular surface; In particular, estrogen because of its use in hormone replace therapy and dexamethasone because of its wide-spread use in ophthalmologic practice, were chosen.

In this study, we examined the effects of estrogen and dexamethasone on the expression of MUC1 and MUC4, the only two known mucins produced by corneal epithelial cells, to determine whether mucin gene expression in ocular surface is influenced by steroid hormones.

## II.Materials and Methods

### 1. Chemicals and reagents

Dexamethasone (DEX), 17β-estradiol, tamoxifen, and mifepristone (RU486) were obtained from Sigma (St. Louis, MO, USA). Dulbecco's modified Eagle's medium was obtained from Gibco-BRL (Rockville, MD, USA), and Ham's F12 from Life Technologies. Fetal calf serum (FCS) (Hyclone, Logan, UT, USA) was treated with dextran coated charcoal to remove glucocorticoids.

### 2. Cells and Culture Conditions

SV-40 transfected human corneal epithelial cells (HCE cells) were kindly provided by Dr Araki-Sasaki K (Toyonaka Municipal Hospital, Osaka, Japan).[19] Cells were cultured at 37°C under a humidified atmosphere of 5 % $CO_2$ and 95 % air. SHEM medium (1: 1 mixture of Dulbecco's modified Eagle's medium and Ham F12 medium, 40 ㎍/㎖ gentamicin, 10 % FCS; DMEM/F12) was used as the basal medium. Cells were grown to confluency in defined medium (DMEM/F12) containing 10% charcoal-stripped calf serum. The medium was replaced with fresh medium without serum and plates were incubated for an additional 2 hours; the medium was removed before adding fresh DMEM/F12 with and without steroid supplement.

### 3. RT-PCR analysis

Total cellular RNA was isolated from human corneal epithelial cells using the Trizol Reagent (Life Technologies, Grand Island, NY, USA) according to the manufacturer's protocol. RT-PCR was carried out using the Perkin Elmer RNA-PCR core kit (Roche Molecular system Inc., Branchburg, New Jersey, USA). The MUC1 human upstream primer was 5-

TCTCACCTCCTCCAATCAC-3 and the downstream primer was 5-GAATGGCACATCACTCAC-3. They were designed to produce a 368 bp PCR product. The PCR conditions were as follows: Initial 5 minutes denaturation at 94°C; 30 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 1 minute, and extension at 72°C for 1 minute; and post-elongation at 72°C for 10 minutes in the Perkin Elmer Gene Amp® PCR system 2400 (Hoff mann-La Roche Inc, Norwalk, CT, USA). For the MUC4, the upstream primer was 5-TTCTAAGAACCACCAGACTCAGAGC-3 and the downstream primer was 5-GAGACACACCTGGAGAGAATGAGC-3. The primers were designed to produce a 467 bp PCR product. This sequence was taken from a region upstream of the repeat sequence of MUC4 that is homologous to the sequence encoding the ASGP-1 mucin subunit of sialomucin. PCR conditions were as follows: denaturation for 5 minutes at 94°C; 40 cycles of denaturation at 94°C for 1 minute, annealing at 53°C for 1 minute, and extension at 72°C for 1 minute; and post-elongation at 72°C for 10 minutes. The 20 $\mu\ell$ of PCR products were electrophoresed on 2% agarose gel containing ethidium bromide.

**4. Immunoblot Analysis**

Human corneal epithelial cells were solubilized in lysis buffer (25 mM HEPES pH 7.5, 0.3 M NaCl, 1.5 mM MgCl2, 0.2mM EDTA, 0.05% Triton X-100, 20 mM b-glycerolphosphate, 1 mM orthovanate, 0.5 mM DTT, 0.4 mM PMSF, 2 $\mu g/m\ell$ leupeptin, 1 $\mu g/m\ell$ pepstain). The Lysate were adjusted to 20 $\mu g$ of total protein per sample and mixed with 5X sample loading buffer (60 mM Tris-HCl pH 6.8, 25% glycerol, 2% SDS, 14.4 mM 2-mercaptoethanol, 0.1% bromophenol blue). The samples were boiled for 5 minutes, and run on 6% separating, 4%

stacking sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The resolved proteins were transferred to Immobilion™-P (Millipore corp., Bedford, MA) PVDF membranes. The membranes were treated with 0.1 U/㎖ of Type V neuraminidase (Sigma Chemical Co., St. Louis, MO, USA), in 50 mM sodium acetate, and 10 mM calcium acetate buffer for 1 hour at 37°C, washed with the Tris-buffered saline and acetate buffer, and blocked in 5%(w/v) non-fat dry milk for 1 hour at room temperature. Membranes were incubated overnight at room temperature with the primary antibody, 1G8, mAb for ASGP-2 (kindly provided by Dr Carraway KL, Dept. of cell biology and anatomy, university of Miami school of medicine, Miami, FL),[20] diluted 1:3000 in 5%(w/v) non-fat dry milk solution or HMFG-1, mAb for MUC1 (Biodesign, Saco, ME, USA)HMFG and then incubated for 1 hour at room temperature with secondary antibody, anti-mouse IgG (Amersham Pharmacia Biotech, Buckinghamshire, England) diluted 1:1000 in 5%(w/v) non-fat dry milk solution.. Between step, the membrane was washed 3 times for 5 minutes each in TBST buffer (Tris buffered saline containing 0.1% Tween-20). Detection was carried out using ECL™ detection reagents (Amersham Pharmacia Biotech) and exposed to the Hyperfilm™ ECL™ (Amersham Pharmacia Biotech).

## III. Results

### 1. Modulation of MUC4 expression by steroid hormones in HCECs

To determine whether steroid hormones may be implicated in the expression of MUC4 in HCECs, we compared the levels of MUC1 and MUC4 mRNA and its protein in the medium supplemented with estrogen or dexamethasone. As shown in Figure 1A, B, estrogen markedly

up-regulated levels of MUC4 mRNA over the control in a time- and dose-dependent manner. To confirm the expression of the corresponding proteins, we extracted cell lysates and performed Western blot analysis. Bands for MUC4 at approximately 80 kDa were detected (Fig. 1B). The results correlated with the RT-PCR data, and demonstrated an increased expression of MUC4 in estrogen treated HCECs compared with non-treated controls.

As shown Figure 2A, the expression of MUC4 was substantially decreased in a time-dependent manner after treating cells with dexamethasone. The decreased levels were maintained during the 36 hour time-course of this experiment. Two days after treatment with different concentrations of dexamethasone, the expression of MUC4 was decreased in a dose-dependent manner even at dexamethasone concentrations as low as $10^{-8}$M (Fig. 2B). A plateau was reached at $10^{-7}$M. This was confirmed by western blot analysis. Cell lysates from the control cells and cells treated with dexamethasone for 48 hours were analyzed by SDS-PAGE. As shown in Figure 2C, lower levels of MUC4 were detected from the lysates of the dexamethasone-treated cells compared with non-treated control cells, suggesting that the MUC4 was down-regulated by dexamethasone.

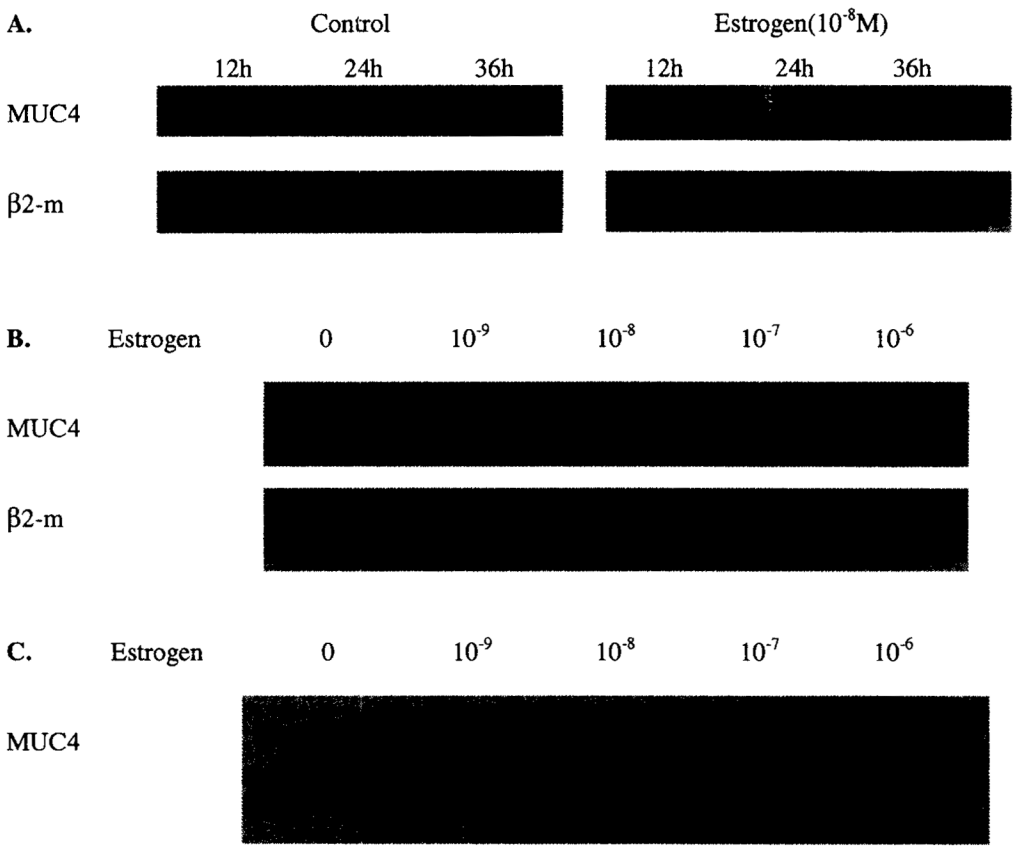


Figure 1. A, Effects of 17β-estradiol ($10^{-8}$M) on MUC4 expression in human corneal epithelial cells. Cells were incubated with 17β-estradiol and RT-PCR analysis for MUC4 mRNA is shown according to length of treatment. B, Effect of 17β-estradiol on MUC4 expression in human corneal epithelial cells according to dose. Cells were incubated with or without 17β-estradiol($10^{-9}$~$10^{-7}$M). RT-PCR analysis shows increased levels of MUC4 mRNA over the control in a dose-dependent manner. C, Expression of MUC4 proteins according to the dose of 17β-estradiol. Bands for MUC4(80 kDa) was demonstrate an increased expression of MUC4 in HCECs treated by estrogen versus non-treated.

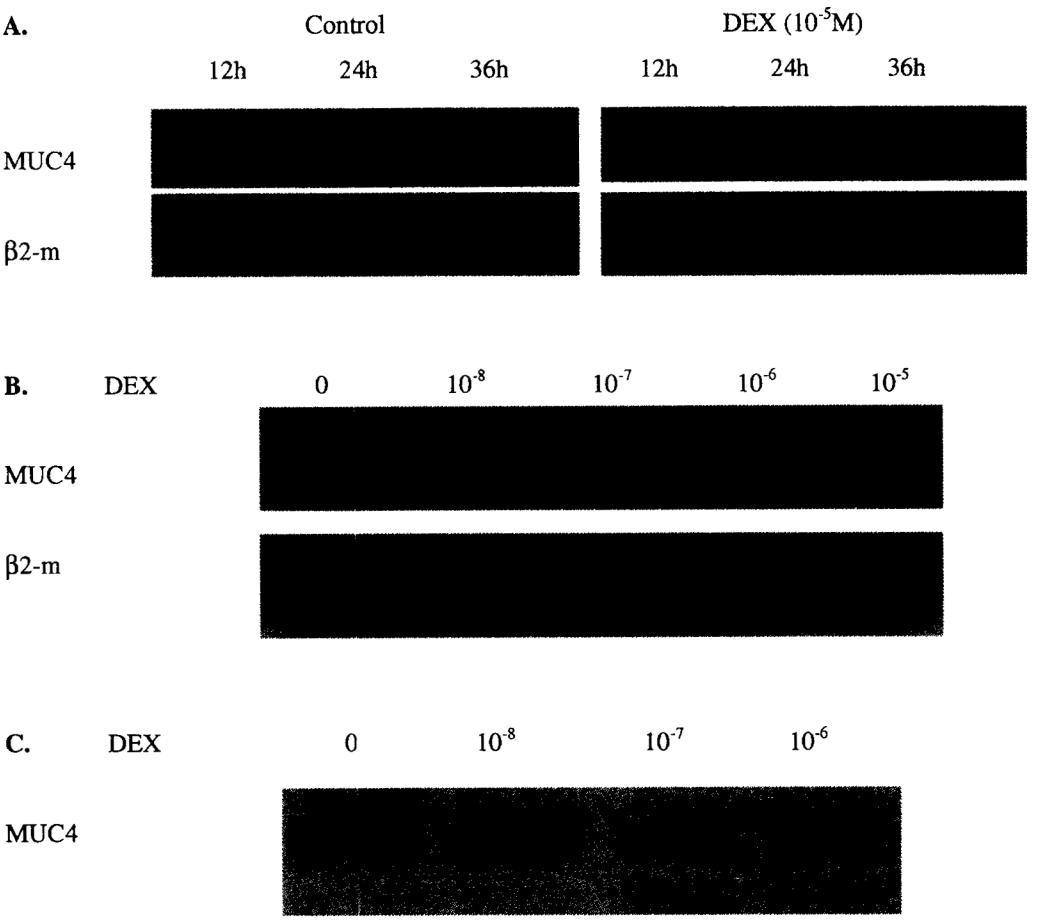


Figure 2. A, Effects of dexamethasone ($10^{-5}$M) on MUC4 expression in human corneal epithelial cells. Significantly decreased level of MUC4 mRNA are shown according to time course. B, Effect of varying dexamethasone concentrations on MUC4 expression in human corneal epithelial cells. The expression of MUC4 is decreased in a dose-dependent manner. C, Modulation of the expression of MUC4 proteins by dexamethasone. Lower levels of MUC4 antibody binding to lysates of the dexamethasone-treated cells compared to control cells suggests that MUC4 is down-regulated by dexamethasone.

**2. Modulation of MUC1 expression by steroid hormones in HCECs**

In contrast to MUC4, the expression of MUC1 mRNA was increased after treatment with dexamethasone (Fig. 3A). In non-treated control cells, only very low levels of MUC1 were detectable. In Western blot analysis to confirm the expression of the corresponding proteins, a much weaker band was observed in the lysates of the control cells compared to dexamethasone-treated cells (Fig. 3B). The expression of MUC1 proteins was increased in a dose-dependent manner apparent at dexamethasone concentrations as low as $10^{-8}$M (Fig. 3B).

As shown in Figure 4A, B, levels of MUC1 mRNA were not affected by estrogen. Similarly in western blot analysis, estrogen did not significantly alter MUC1 protein levels (Fig. 4C).

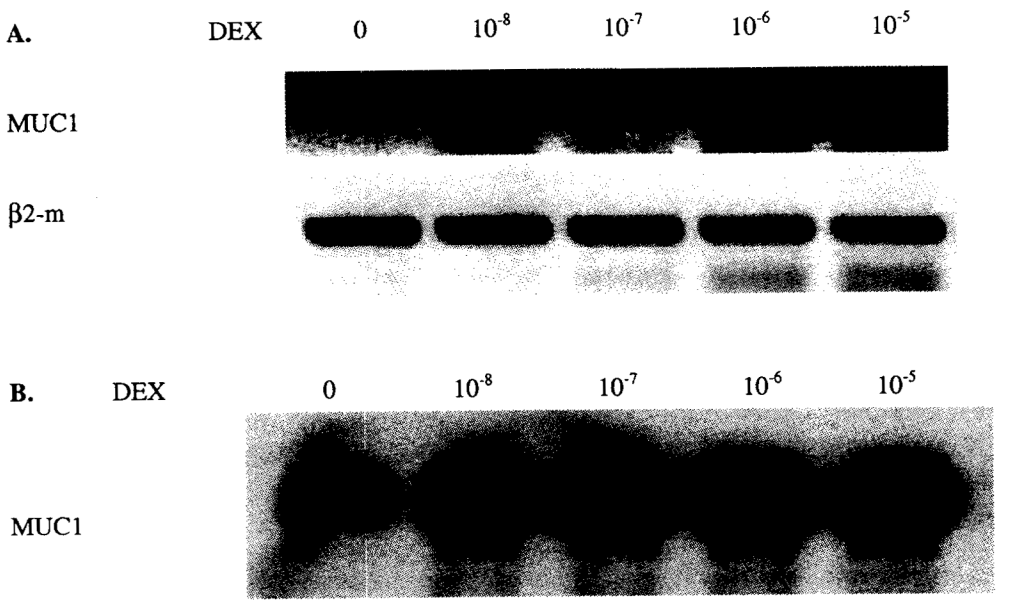


Figure 3. A, Effects of dexamethasone($10^{-5}$M) on MUC1 expression in human corneal epithelial cells. Upregulation of MUC1 mRNA by dexamethasone is shown according to time course. B, Effect of various dexamethasone concentrations on MUC1 expression in human corneal epithelial cells. The expression of MUC4 is increased in dexamethasone-treated cells compared with control cells. C, Modulation of the expression of MUC1 proteins by dexamethasone. Levels of MUC1 antibody binding increases in the lysates of the dexamethasone-treated cells compared with control cells.

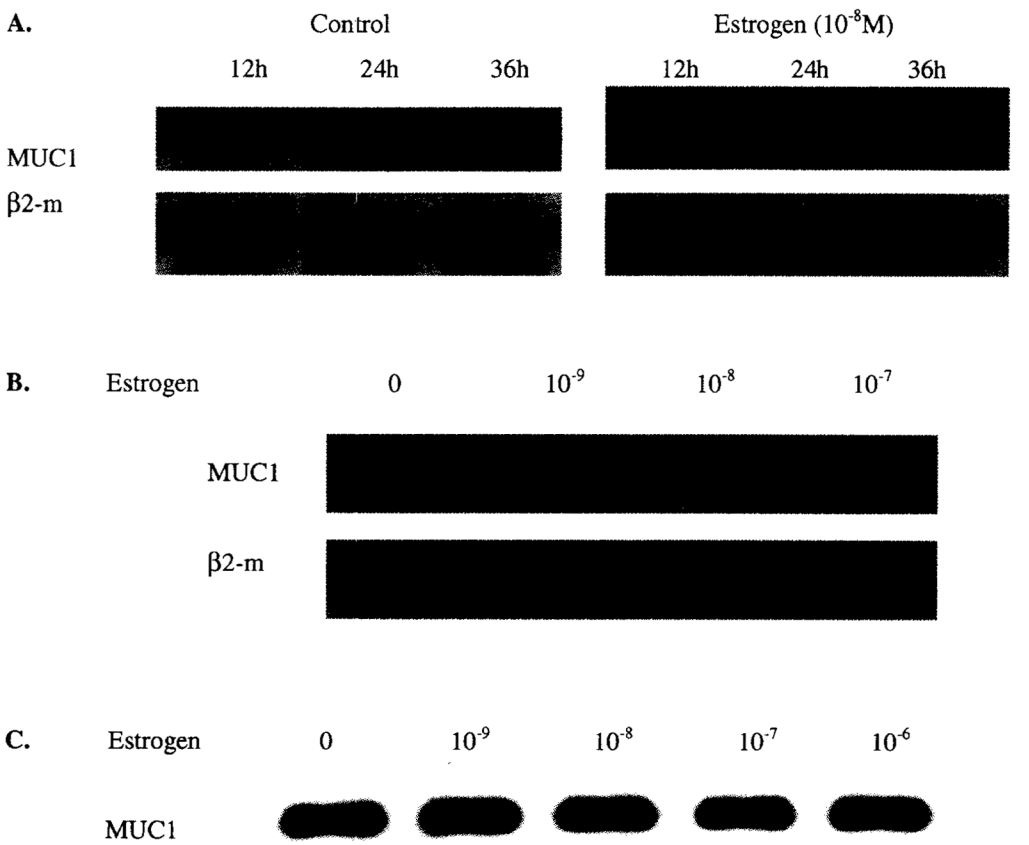


Figure 4. A, Effects of 17β-estradiol ($10^{-8}$M) on MUC1 expression in human corneal epithelial cells. B, Effect of 17β-estradiol on MUC1 expression in human corneal epithelial cells according to dose. RT-PCR analysis shows constant levels of MUC4 mRNA. C, Expression of MUC4 protein according to the dose of 17β-estradiol. MUC4 protein (80 kDa) levels were not affected by treatment with estrogen.

**3. Receptor antagonists inhibited the effects of steroid hormones on mucins in HCECs**

To further investigate whether estrogen is directly involved in the induction of MUC4, HCECs were cultured for 48 hours in the following conditions: estrogen ($10^{-8}$M), estrogen plus the estrogen receptor antagonist, tamoxifen ($10^{-7}$M), and no treatment (control). The addition of tamoxifen, which is a selective irreversible inhibitor for estrogen, to the cells treated with estrogen led to a considerable reduction in expression of MUC4 (Fig. 5). These results further demonstrated that the stimulatory estrogen effect was estrogen receptor-mediated.

To determine if dexamethasone's effect on MUC4 was glucocorticoid receptor-mediated, HCECs were cultured for 48 hours, in the presence of dexamethasone ($10^{-6}$M), or in the presence of dexamethasone plus the glucocorticoid receptor antagonist (RU486), or with no treatment (control). Total cellular RNA was extracted and subjected to an RNase protection assay with antisense riboprobes corresponding to MUC4. As shown in Figure 6, treatment with dexamethasone resulted in significant inhibition of MUC4 protein expression. However, the concurrent treatment with RU486 ($10^{-5}$M) resulted in a substantial (albeit incomplete) reversal of that effect thereby, suggesting that the inhibitory effect of dexamethasone is glucocorticoid receptor-mediated.

To further confirm that the dexamethasone effect on MUC1 was glucocorticoid receptor-mediated, HCECs were cultured in the absence of treatment, in the presence of dexamethasone ($10^{-6}$M), or in the presence of dexamethasone plus the glucocorticoid receptor antagonist (RU486). Cell lysates from the control cells and cells treated for 48 hours were analyzed by SDS-PAGE. As shown (Fig. 6), the increased expression of MUC1 protein mediated by

dexamethasone was reversed by concurrent treatment with RU486 to a level indistinguishable from control. This further suggests that MUC1 expression may be mediated by glucocorticoid receptors.

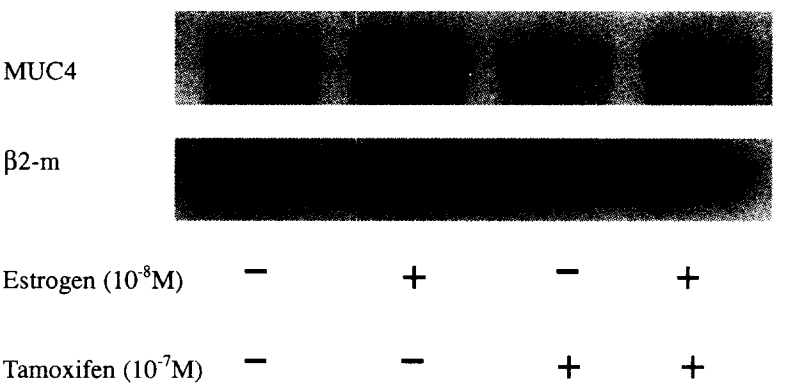


Figure 5. Effect of tamoxifen on 17β-estradiol-stimulated MUC4 in human corneal epithelial cells. Cells were incubated with or without 17β-estradiol ($10^{-8}$M) and/or tamoxifen ($10^{-7}$M) for 48 h.

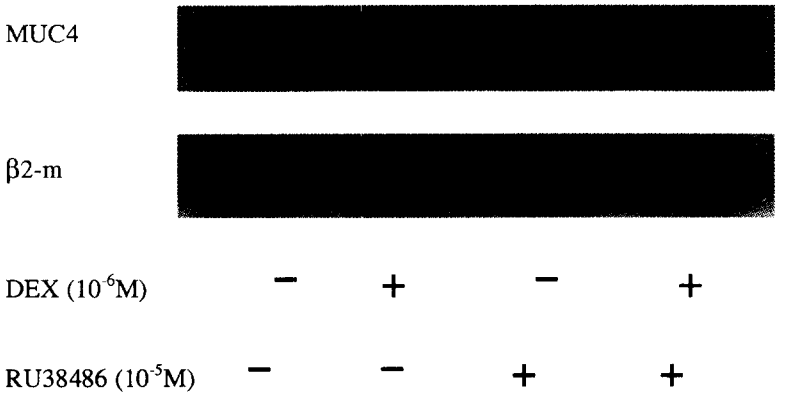


Figure 6. Effect of RU38486 on dexamethasone-induced MUC4 suppression in human corneal epithelial cells. Cells were incubated with or without dexamethasone ($10^{-6}$M) and/or RU38486 ($10^{-5}$M) for 48 h.

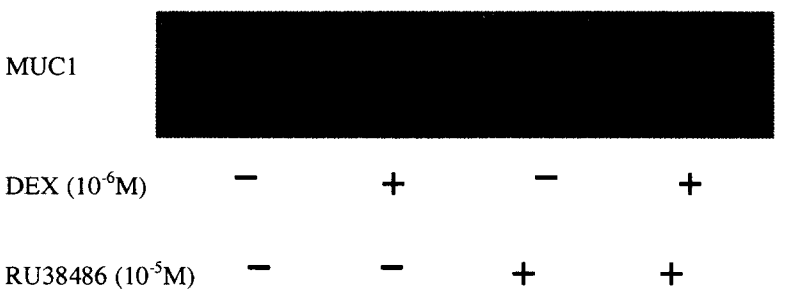


Figure 7. Effect of RU38486 on dexamethasone-induced MUC1 expression in human corneal epithelial cells. Cells were incubated with or without dexamethasone ($10^{-6}$M) and/or RU38486 ($10^{-5}$M) for 48 h.

## IV. Discussion

The modulation of mucin by steroids hormones has been previously characterized in various cell types including the breast cancer, multiple myeloma, human alveolar epithelial, human endometrial cancer, and human gastric mucosal cells. In this process, the consensus sequences of promoters of the MUC1 and MUC4 genes, for the binding of estrogen, progesterone or glucocorticoid receptor complexes has been implicated. [6-11] The results presented here indicate for the first time that the mucins are regulated by steroid hormones in human corneal epithelial cells and this may be mediated by gene specific transcriptional control of steroid hormones. We showed that the expression of MUC1 was significantly augmented and that of MUC4 was significantly suppressed in the dexamethasone-treated HCECs. Estrogen up-regulated the levels of MUC4 over the control levels but not those of MUC1. The steroid hormone effects on the mucins were reversed by the respective receptor antagonists.

The presence of estrogen, progesterone and glucocorticoid receptors in corneal epithelial cells has been reported previously.[21-26] Our RT-PCR and western blot analysis showed the blockage of these receptors abolished the effects of steroid hormones on the mucin expression in corneal epithelial cells, confirming transcriptional roles of these receptors. However, the effect of estrogen receptor antagonist was as apparent as that of glucocorticoid receptor antagonist. A possible explanation for this discrepancy is the partial agonist effect of the tamoxifen on the estrogen receptor. [27-29] The relative levels of ERα and ERβ are an important determinant of this activity; ERß is known to suppress the partial agonist activity of tamoxifen on ERα.[30] Recent reports suggested that occurrence of ERβ in human cornea, contrary to that of rodent,[21] is very

low to zero. Thus, it is possible that the partial agonist activity of tamoxifen on ERα is not suppressed in human corneal epithelial cell.

The effects of replace therapy on dry eye in postmenopausal women has been previously studied.[31, 32] Estrogen as topical eyedrops has been reported to significantly improve all observed ocular symptoms and Schirmer's test values of dry eye in menopausal women. However, systemic hormone replace therapy by estrogen has no effects on dry eye.[32] Sator et al., suggested that the "blood-eye barrier" prevents systemic estrogens from acting on the ocular surface. The improvement of dry eye by estrogen could be caused by the changes in the corneal and conjunctival condition, as opposed to changes in the lacrimal gland, which has no blood barrier. Our finding of increased expression of MUC4 by estrogen in the HCECs may be a possible explanation for the effect of topical estrogen on the postmenopausal dry eye.

Notably, glucocorticoid hormone has also been implicated in the production of mucin in human ocular surface, especially in conjunctival epithelial cells.[33] A thick mucus layer was observed when hydrocortisone was present in the culture medium; however, that layer was absent in cultures grown with hydrocortisone-free media in that study using electron microscopy. However, in corneal epithelial cells, there has as yet been no report regarding the influence of glucocorticoid hormone on the mucin production. Our findings that dexamethasone-mediated reduction of MUC4 and increased expression of MUC1 observed in HCECs suggests that dexamethasone may have the opposite effects on the production of mucins in corneal epithelial cell, and we believe that additional studies of the overall effect of this should help to further delineate further its role in ocular surface production of mucin.

Immunofluorescence data from recent reports suggested that MUC4 was more heavily concentrated in the superficial layers of corneal epithelium and MUC1 appeared to be more abundant in the basal layers. It has been proposed that they have distinctly different functions based on their locations.[18, 34] Herein, we have shown that steroid hormones regulate mucin expression of corneal epithelial cells. In order to definitively delineate these effects of steroid hormones on corneal epithelial cell according to their location and mucin type, and to understand the role of the modulation of mucin on tear film, further investigation must be performed using the multi-layered corneal epithelial cell model.

## V. Conclusions

In conclusion, we report that steroid hormones regulate mucin expression in human corneal epithelial cells: Estrogen increased MUC4 on the transcriptional level, dexamethasone suppressed MUC4 on the transcriptional level, and dexamethasone increased MUC1 on the transcriptional level.

Moreover, this expression of mucin might be mediated by the respective steroid hormone receptors: Estrogen-related expression of MUC4 was shown to be mediated through the estrogen receptor, dexamethasone-related suppression of MUC4 through the glucocorticoid receptor, and dexamethasone-related expression of MUC1 also through the glucocorticoid receptor.

Although little is known about the differentiation-dependent biosynthesis, glycosylation, intracellular processing, and secretion of mucins by the ocular surface epithelium, even less is known about the control of mucin expression in the ocular surface. In this study, we have shown for the first time that steroid hormones regulate mucin expression of corneal epithelial cells and that their respective hormone receptors mediate this modulation of mucin expression.

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**국문요약**

스테로이드 호르몬이 각막상피세포 뮤신의 발현증감에 미치는 영향

뮤신은 안구표면의 유지와 보호에 중요한 눈물층 성분의 하나이다. 안구표면의 뮤신으로는 각막과 결막 상피의 세포막 뮤신인 MUC1, MUC4 와 결막 배상세포의 분비 뮤신인 MUC5AC 이 생성되는 것으로 알려져 있다. 하지만, 이와 같은 안구표면의 뮤신을 어떻게 조절할 수 있는지에 대해서는 알려진 바가 없다. 본 연구에서는 안구표면 중 자각증상과 시력에 중요한 각막의 상피세포에서 뮤신이 스테로이드 호르몬에 의해 변화하는지 알아보고자 하였다.

인체 각막상피세포(HCEC)에 덱사메타존과 에스트로겐을 처치하여 MUC1 과 MUC4 의 변화를 농도별, 시간별로 관찰하였다. 단백질의 양은 immunoblot 을 이용하여 비교하였고, mRNA 는 RT-PCR 로 비교하였다.

MUC4 는 덱사메타존을 처치하였을 때 농도와 시간에 따라 감소하고, 에스트로겐 처치시에는 농도와 시간에 따라 증가함을 알 수 있었다. MUC1 은 에스트로겐에 의해서는 농도와 시간에 따라 변화를 보이지 않았던 반면, 덱사메타존에 의해서는 증가하였다. 덱사메타존의 MUC4 감소 효과와 MUC1 증가 효과는 부신피질호르몬 수용체 길항제인 RU486 에 의해 차단되었고, 에스트로겐에 의한 MUC4 의 증가 효과는 에스트로겐 수용체 길항제인 tamoxifen 에 의해 감소되었다.

본 연구를 통해 스테로이드 호르몬은 각막 상피 세포에서 뮤신의 생성에 관여하며, 이와 같은 작용에 각 호르몬 수용체가 관여함을 알 수 있었다.

핵심 되는 말 : 에스트로겐, 덱사메타존, MUC1, MUC4, 각막상피세포