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Cellular Proliferative Effect of Dexamethasone in Immortalized Trabecular Meshwork Cell (TM5) Line

Jae Won Jeon[1,2], Seung Jae Lee[1,2], Jong Bin Kim[1,2], Jimmy Jaeyoung Kang[1,2], Joon Haeng Lee[1,2,3], Gong Je Seong[1,2], and Eung Kweon Kim[1,2,3]

[1]Department of Ophthalmology, [2]Institute of Vision Research, [3]BK21 Project for Medical Science, Yonsei University College of Medicine, Seoul, Korea.

Dexamethasone (DEX), one of the corticosteroid hormones, is one of the most common therapeutic strategies in ophthalmological treatment. Despite its widespread use and clinical efficiency, little is known regarding the specific effects of DEX on cell growth, differentiation and cell death in human trabecular meshwork cells. The presence of the glucocorticoid receptor (GR, dexamethasone receptor) in TM-5 cell line, which was derived from the primary human trabecular meshwork cells, was verified by RT-PCR and western blot analysis. The effects of DEX on the cellular proliferation of TM5 cells were measured by a BrdU incorporation assay. Western blot analysis were used to examine the effects of DEX on the Ras/MEK/ERK signaling pathway. The total Ras, MEK1/2 and ERK1/2 protein levels as well as the levels of activated (phosphorylated) form were both significantly increased by the DEX treatment for 5 days. Both MEK1/2 and ERK1/2 were significantly activated by phosphorylation after 10 minutes. The dependence of this increased cell proliferation on GR activation by DEX and the sustained activation of ERK was examined using RU486 (a GR inhibitor) and U0126 (a MEK inhibitor). Both RU486 and U0126 prevented the induction of cell proliferation by the DEX treatment in the TM5 cells. In conclusion this study demonstrated that GR is expressed in TM5 cells. Secondly, DEX treatment for 5 days stimulates cell proliferation in TM5 cells, and that this increased proliferation effect is mediated by the Ras/MEK/ERK pathway.

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Reprint address: requests to Dr. Eung Kweon Kim, Department of Ophthalmology, Institute of Vision Research, Yonsei University College of Medicine, 134 Shinchon-dong, Seodaemun-gu, Seoul 120-752, Korea. Tel: 82-2-361-8450, Fax: 82-2-312-0541, E-mail: eungkkim@yumc.yonsei.ac.kr

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INTRODUCTION

The trabecular meshwork (TM) is a major regulation site of the normal bulk flow of the aqueous humor.[1] The pathophysiology of TM cells have been studied in various ways because a dysfunction or alteration of the TM cell activity may be responsible for the development of glaucoma.

Dexamethasone (DEX) is a glucocorticoid commonly used after cataract surgery, penetrating keratoplasty and refractive surgery in order to reduce ocular inflammation or to delay corneal wound healing.[2] However, the administration of the instillation of DEX results in an increase in the intraocular pressure in certain patients, which can cause vision damage. The exact relationship between the instillation of steroids and the increase in the intraocular pressure is not known.

The glucocorticoid receptor (GR) is a member of the steroid receptor super-family that mediates the physiological processes controlled by glucocorticoids. GR activates transcription by binding to the glucocorticoid response elements (GREs), and represses transcription by binding to the negative and composite elements.[3]

It has been reported that DEX treatment on cultured human TM cells results in an enlargement of the cells without proliferation.[4,5] However, low concentrations of DEX have been shown to induce cell proliferation on cultured human corneal keratocytes and corneal epithelial cells.[6,7] The TM5

cell is one of the immortalized human TM cells. This study investigated the cellular effects of DEX in the TM5 cells as a way of determining the various characteristics of TM cells by examining its effects on cell proliferation *in vitro* and its modulation of the Ras/MEK/ERK pathway.

MATERIALS AND METHODS

Cell culture

TM5 cells were obtained from Alcon Research Ltd (Fort Worth, TX, USA). The TM5 cells were maintained in Dulbecco's Modified Eagle's Medium (DMDM, GibcoBRL, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS, Gibco BRL), 50 ug/ml streptomycin (Sigma Chem co., St. Louis, MO, USA), 50 ug/ml penicillin (Sigma), at 37℃ in 5% CO_2. The medium containing charcoal-treated 10% FBS in order to eliminate steroids in the serum was added to the TM5 cells for DEX treatment.[8]

Drug preparation and treatment

DEX (Sigma) was dissolved and serially diluted in absolute ethanol prior to its addition to the culture medium. The medium was replaced with 3 ml of complete medium containing various concentrations of DEX (10^{-9}, 10^{-7} or 10^{-5} M) in 60 mm culture dishes. DEX was added to the culture medium every 2 days at the same concentration. In all experiments, the ethanol concentration in the culture media was maintained at 0.1%. All the solutions were filter sterilized and stored at 4℃ in light-proof containers. The control group consisted of TM5 cells cultured in SHEM with 0.1% absolute ethanol without DEX. The culture medium was renewed every 2 days.

Experiment for the presence of GR in TM5 cells

Isolation of total RNA

The total RNA was isolated from the TM5 cells by using RNeasy Mini Kit (Qiagen, Courtaboeuf, France). The disruption and homogenization of the samples were performed using a QIAshredder (Qiagen). The quantity of extracted RNA was quantified by measuring the absorbance at 260 nm. The purity of the RNA was confirmed by the ratio between the absorbance values at 260 and 280 nm, which ranged between 1.77 and 2.11, demonstrating the high quality of the RNA. This was confirmed by electrophoresis of the RNA on a 1.5% agarose gel containing ethidium bromide.

RT-PCR

The Oligo $(dT)_{15}$-primed first strand cDNA was synthesized using an RT-PCR kit (Roche, Branchburg, New Jersey, USA) according to the supplier's instructions. In order to eliminate the residual genomic DNA from the RNA samples, 2 μg of the isolated RNA was incubated with 1 unit of RQ1 DNase (Promega, Madison, Wisconsin, USA) in a 10 μl reaction volume containing DNase buffer (400 mM Tris-HCl at pH 7.5, 60 mM $MgCl_2$, and 100 mM NaCl) at room temperature for 15min. After heat-inactivation of the RQ1 DNase at 65℃ for 15 min, half of the reaction mixture was used for RT, and the other half was used for the negative control reverse transcription. The cDNAs were synthesized from 1 μg of DNase-treated RNA in a 20 μl reaction containing 5 mM $MgCl_2$, RT Buffer (10 mM Tris-Cl at pH 9.0, 50 mM KCl, 0.1% Triton X-100), 1 mM each dNTP, 1 unit of a Recombinant RNasin Ribonuclease Inhibitor, and 0.5 μg of the Oligo $(dT)_{15}$ primer with or without 1.5 units of MuLV reverse transcriptase at 42℃ for 1 h. PCR was performed using Ex Taq polymerase (Takara, Shiga, Japan) under the following conditions: 95℃ for 15 sec, 5 7℃ for 30 sec, 72℃ for 1 min for 25 cycles. The primer set used for GR was 5'-ATGAGACCAGA TGTAAGCTC (forward) and 5'-AATGCCATAAG AAACATCCA (reverse).[6] The reaction products were analyzed on 1.2% agarose gel.

Western blot analysis

The cells were washed twice with phosphate buffered saline (PBS), scraped from the culture vessels, and then collected. The harvested cells were suspended and disrupted on ice in 250 μl of lysis buffer [10 mmol/L Tris, pH 7.4; 1 mmol/L EDTA; 10% sodium dodecyl sulfate (SDS); 1% glycerol; 1 mg/ml leupeptin; 1 mg/ml pepstatin; 50 mg/ml aprotinin and 0.5 mmol/L phenylmethysulfonyl fluoride; all obtained from Sigma].

Equal amounts of the sample proteins were electrophoretically separated on 10 or 12% SDS gel. The separated proteins were electrically transferred to nitrocellulose membranes, and the membrane was incubated for 1 h in a blocking solution (5% nonfat dry milk in Tris-buffered saline /0.1% Tween20 (TBS-T). Subsequently, GR antibodies (SantaCruz, CA, USA) were added and incubated for 2 h at room temperature. Subsequently, the blot was washed several times in TBS-T, and incubated for 1 h with a 1 : 1000 dilution of sheep anti-rabbit Ig horseradish peroxidase-conjugated secondary antibodies (Amersham, Uppsala, Sweden). The reactive bands were visualized using the ECL-enhanced chemiluminescence method (Amersham).

Experiment for the translocation of GR into nucleus after DEX treatment

The TM5 cells treated with or without DEX for 10 minutes were rinsed with PBS, fixed at room temperature for 15 min in a 4% paraformaldehyde solution (Sigma) and washed with PBS/0.1% Tween20 (PBS-T). The non-specific sites were blocked by incubating the cells at room temperature in a 10 % NGS in PBS for 1 h. The cells were incubated with the anti-GR antibodies for 2 h at room temperature and washed with PBS-T for 30 min. The cells were then incubated with goat anti-rabbit Ig conjugated to FITC (Sigma) at 1/100 dilution in the PBS-T solution for 1 h at room temperature. After three PBS washes, the cells were labeled for 10 min with 100 μg/ml propidium iodide (Sigma) in order to label the nuclei, and the coverslips were mounted and analyzed using a Olympus Fluorescence microscope (BX60) under the appropriate fluorescence filter blocks. The images were recorded on Fuji color film (ASA 400).

Experiment for the effect of DEX treatment on cell proliferation

Bromodeoxyuridine (BrdU) Incorporation Assay

The effect of DEX on the TM5 cell proliferation was examined by a BrdU incorporation assay. For BrdU labeling, the TM5 cells treated with or without DEX for 3 days were plated to 30% confluence on 2 well chamber slide dishes (NUNC, Naperville, IL, USA). After 48 h, 10 μM of BrdU (Sigma) was added to the medium for 5h. The BrdU-treated cells were rinsed with PBS, fixed at room temperature for 15 min in a 4% paraformaldehyde solution (Sigma) and washed with PBS-T. The cells were treated with 2 N HCl for 1h. After several washes with H_2O and PBS, the non-specific sites were blocked by incubating the cells at room temperature in 10% normal goat serum in PBS for 1 h. The cells were then incubated for 30 min at room temperature with mouse anti-BrdU antibodies (Sigma), washed three times with PBS and incubated with donkey anti-mouse Ig conjugated to FITC (Sigma) at a 1/100 dilution in the PBS-T solution for 1 h at room temperature. Propidium iodide staining and image recording were carried out as described above.

Western blot analysis with antibodies for erk pathway

The well-characterized ERK pathway was examined in order to elucidate the mechanisms for the induction of cell proliferation. All procedures were the same as described above. The following antibodies were purchased from Transduction Laboratories (San Diego, CA, USA): anti-Ras, c-Raf-1 and MAP kinase Phosphatase 2 (MKP2). Anti-phospho-Raf, phospho-MEK1/2, phospho-ERK1/2, MEK1/2 and ERK1/2 antibodies were purchased from Cell Signaling (Beverly, MA, USA). The anti β-actin monoclonal antibodies were purchased from Sigma.

The effects of RU486 (GR inhibitor) and U0126 (MEK1/2 inhibitor) were examined in order to study the dependence of DEX-induced cell proliferation and the activation of ERK pathway on GR and on the sustained activation ERK.

RESULTS

Experiment for the presence of GR

RT-PCR of the total RNA from TM5 cells resulted in a single band of the expected length of 640 bp for GR. The positive control PCR reaction of the total RNA obtained from the human

corneal epithelium, in which GR is known to be expressed,[7] also yielded a product of an identical size (Fig. 1A). Western blot analysis of the lysates obtained from the TM5 cells and human corneal epithelial cells confirmed the presence of the corresponding protein (Fig. 1B).

Experiment for the translocation of GR into nucleus after DEX

Immunofluorescence analysis showed the anti-GR antibody response only from the cytoplasm prior to the DEX treatment. The anti-GR antibody response was detected not only in the cytoplasm but also in the nucleus 10 minutes after the DEX treatment (Fig. 2).

The effect of DEX treatment on cell proliferation

Brdu incorporation assay

A higher level of BrdU incorporation was observed in the DEX treated cells at concentrations as low as 10^{-9}M compared to the untreated controls indicating enhanced cell proliferation (Fig. 3). The number of BrdU-labeled cells was the highest at a DEX concentration of 10^{-7}M with a slightly lower number of labeled cells observed at a DEX concentration of 10^{-5}M.

Activation of the Ras/Raf/ERK pathway by DEX treatment

Treating the TM5 cells with DEX for a period of 5 days resulted in higher amounts of Ras, MEK1/2, and ERK1/2. The amounts of activated

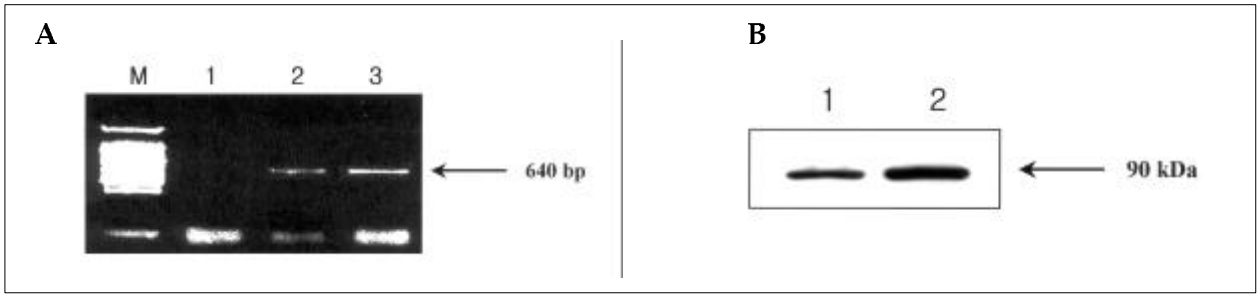


Fig. 1. GR expression in TM5 cells using RT-PCR (A) and western blot analysis (B). (A) A single band of the expected length of 640bp for GR was obtained by RT-PCR of the total RNA from the TM5 cells. M; marker, lane 1; negative control (without reverse transcriptase), lane 2; TM5 cells, lane 3; human corneal epithelium (HCE; positive control). (B) The cell lysates from the TM5 cells and HCE were separated on 8% SDS-PAGE gel and blotted with anti-GR antibodies. A 90kDa band was detected on both cells. Lane 1; TM5 cells, lane 2; HCE (positive control).

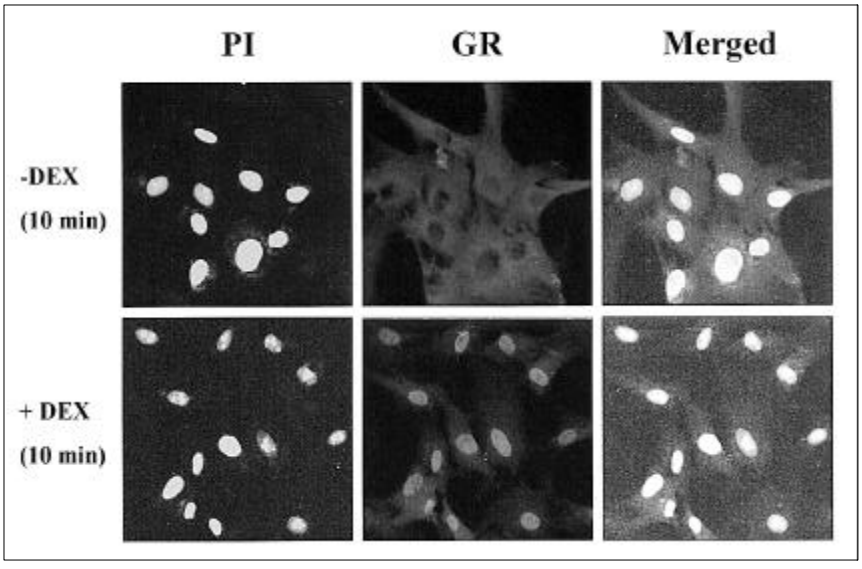


Fig. 2. Activated GR translocation was observed by immunofluorescence staining using anti-GR antibodies. Activated GR translocated into the nucleus after the DEX treatment for 10 minutes (+DEX), whereas the inactivated GR without DEX (-DEX) did not translocate and was present in the cytoplasm.

MEK1/2 and ERK1/2 were also increased with the DEX treatment. While the c-Raf protein levels were unchanged with DEX treatment, the level of activated (phosphorylated) c-Raf was increased.

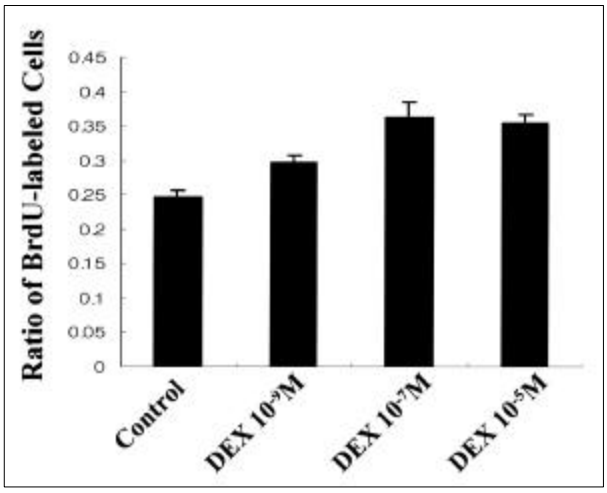
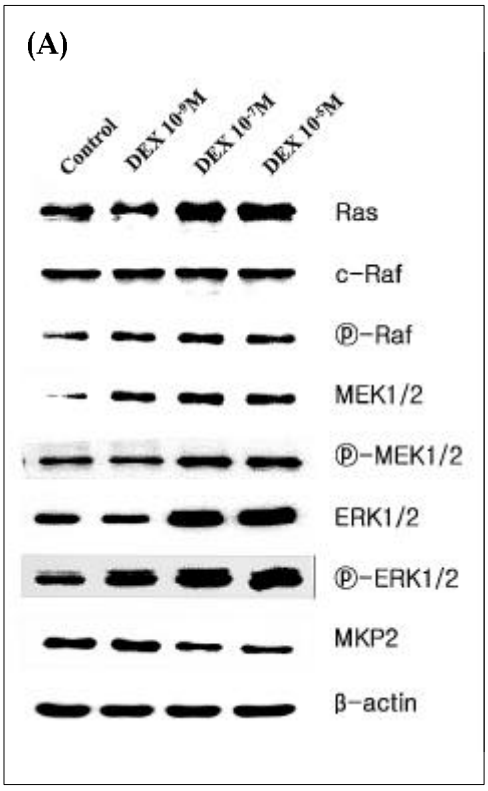


Fig. 3. TM5 cell proliferation analyzed by a BrdU incorporation assay. The bars (mean ± SD; n=3) represent the proportions of BrdU positive cells obtained from three independent experiments and two different persons. Note that the maximum proliferative effect was observed at 10^{-7}M.

This activation of the ERK pathway was the greatest at a DEX concentration of 10^{-7}M and was slightly lower at a concentration of 10^{-5}M. This was in agreement with the results of the BrdU incorporation assay. It should also be noted that MAP kinase phosphatase 2 (MKP2) protein level, which is known to de-phosphorylate MAPK, was lower after the DEX treatment (Fig. 4A).

The above results were obtained from the relatively long-term (5 days) treatment of the TM5 cells with DEX. Therefore, the short-term effects of DEX were also investigated because of the possibility that activation of the ERK pathway was merely a secondary consequence of the increased cell proliferation induced by other pathways. Both MEK1/2 and ERK1/2 activation were significantly increased after the DEX treatment for only 10 minutes. This sharp increase in MEK1/2 and ERK1/2 activity observed at 10 minutes of treatment was followed by a gradual decrease in activity up until a period of 3 hours of treatment. However, after 1 day of DEX treatment, the level of MEK1/2 and ERK1/2 activation was maintained at constant levels (Fig. 4B).

From the inhibitor assay, RU486 prevented the



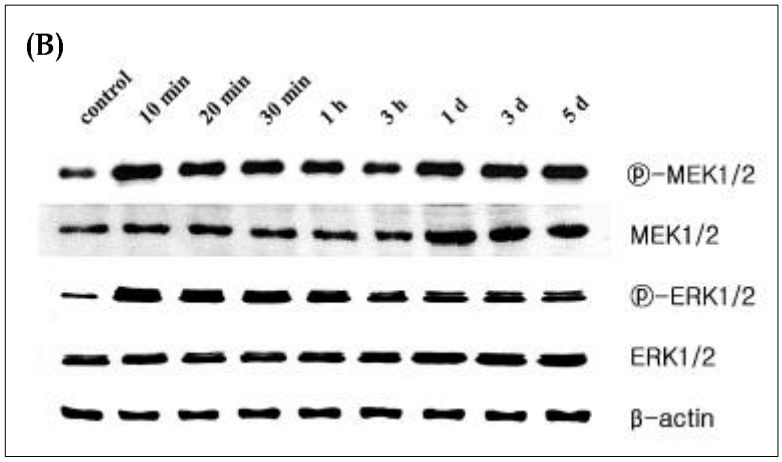


Fig. 4. Dose-dependent (A) and time-dependent (B) effects of DEX treatment for 5 days on MEK/ERK pathway. (A) The same TM5 cells described in Fig. 3 were investigated using antibodies against the molecules involved in MEK/ERK pathway. The cell extracts were fractionated on 10% SDS-PAGE. All molecules except c-Raf were quantitatively induced and activated by DEX, particularly on the concentration of 10^{-7} M, correspondent with the cell proliferation assay shown in Fig. 3. The β-actin was used as the quantitative standard. (B) Note that MEK and ERK were immediately activated (phosphorylated) by DEX (10^{-7}M) treatment for only 10 minutes. The amounts of MEK and ERK were higher and sustained for 5 days compared to the control.

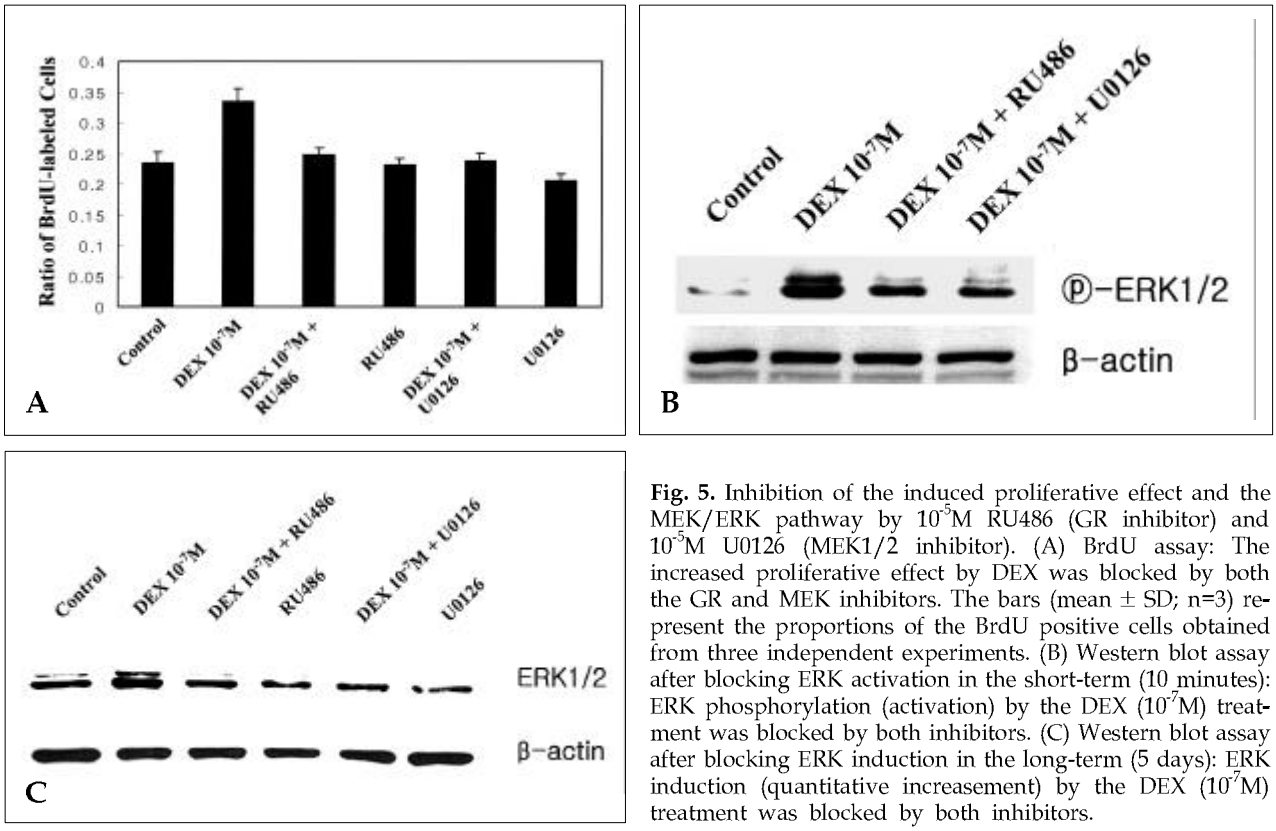


Fig. 5. Inhibition of the induced proliferative effect and the MEK/ERK pathway by 10^{-5}M RU486 (GR inhibitor) and 10^{-5}M U0126 (MEK1/2 inhibitor). (A) BrdU assay: The increased proliferative effect by DEX was blocked by both the GR and MEK inhibitors. The bars (mean ± SD; n=3) represent the proportions of the BrdU positive cells obtained from three independent experiments. (B) Western blot assay after blocking ERK activation in the short-term (10 minutes): ERK phosphorylation (activation) by the DEX (10^{-7}M) treatment was blocked by both inhibitors. (C) Western blot assay after blocking ERK induction in the long-term (5 days): ERK induction (quantitative increasement) by the DEX (10^{-7}M) treatment was blocked by both inhibitors.

induction of cell proliferation by the DEX treatment in TM5 cells. U0126 also prevented the DEX mediated induction of cell proliferation (Fig. 5A). Treatment with either RU486 or U0126 also prevented the DEX-mediated increase in the ERK protein levels as well as ERK activation in the short-term (10 min, Fig. 5B) and ERK activation in the long-term (5 days, Fig. 5C).

DISCUSSION

The data in this study show that (i) GR is present in the TM5 cells (Fig. 1 and 2), (ii) DEX stimulates cell proliferation in the TM5 cells (Fig. 3) and (iii) the GR dependent-activation of the Ras/ MEK/ERK pathway is responsible for this increased proliferative effect (Fig. 4 and 5).

GR protein expression was noted in the TM5 cells (Fig. 1), as was also found in the human corneal epithelial cells.[7] The translocation of GR into the nucleus (Fig. 2) was immediate after the administration of DEX, as reported previously in A6, L929 and DU145 (prostate cancer cell line) cells.[9-12] These results suggest that the effect of DEX on the TM5 cells begins immediately (<10 min) after exposure to DEX.

The increased cell proliferation was detected by a BrdU incorporation assay (Fig. 3) after the DEX treatment. With the increased cellular proliferation, the ERK pathway was examined as a possible signal pathway. Fig. 4A shows that the dose-dependent DEX treatment for 5 days induced most of the molecules involved in the ERK pathway. However, in the experiment investigating the time-dependent treatment of DEX (Fig. 4B), the levels of activated (phosphorylated) MEK1/2 and ERK1/2 increased dramatically within a period of 10 minutes of DEX treatment and then slowly decrease thereafter up to 1 day after the treatment. Subsequently, a second and sustained activation of MEK1/2 and ERK1/2 was observed (Fig. 4B). The Ras, MEK1/2, and ERK1/ 2 protein levels were higher after one day. Five days after DEX treatment, the Ras, MEK1/2, and ERK1/2 protein levels remained high compared to the

untreated controls (Fig. 4B). This suggests that DEX elicits an immediate short term effect in the cytoplasm and a long-term effect via the upregulation of protein expression involved in the MEK/ERK pathway after translocation into the nucleus.[13-16]

The dependence of the actions of DEX on GR and the sustained activation of ERK were investigated using the GR inhibitor, RU 486 and MEK inhibitor U0126. Both inhibitors effectively abolished DEX-mediated induction of TM5 cell proliferation (Fig. 5A). Moreover, the short term activation (Fig. 5B) as well as the long-term activation (Fig. 5C) of ERK1/2 was also prevented in the presence of either RU 486 or U0126. These results strongly suggest that DEX exerts its cell proliferative actions via an interaction with the GR, first in the cytoplasm resulting in the short-term activation of the ERK pathway, followed by its nuclear translocation with the subsequent induction of gene expression of the related ERK molecules in a long-term manner.[17,18]

As glucocorticoids such as DEX are known to cause a form of open angle glaucoma, which is similar to primary open angle glaucoma and is associated with the increased resistance to aqueous humor outflow,[19-21] this *in-vitro* study provides one working hypothesis showing that the administration of DEX may inhibit aqueous humor outflow via the stimulation of cell proliferation in the TM region. One recent report showing proliferation of fibrous connective tissue in the inner wall of the Schlemm's canal among the patients with congenital glaucoma support this hypothesis.[22,23]

Further studies are needed to answer certain critical questions. These include (i) whether this increased proliferation of the TM cells also occurs after DEX treatment *in vivo*; (ii) whether there is a direct relationship between the increased cell proliferation and the induced increase in the intraocular pressure and (iii) how all of these factors may ultimately lead to the development of glaucoma. It is believed that these results will make a contribution to the understanding of the cause and lead to possible new therapies for open angle glaucoma.

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