ERK1/2 pathway mediates epithelial-mesenchymal transition by cross-interacting with TGFβ/Smad and Jagged/Notch signaling pathways in lens epithelial cells

XIAOYUN CHEN*, SHAOBI YE*, WEI XIAO, WENCONG WANG, LIXIA LUO and YIZHI LIU

State Key Laboratory of Ophthalmology, Zhongshan Ophthalmic Center, Sun Yat-sen University, Guangzhou, Guangdong 510060, P.R. China

Received February 6, 2014; Accepted April 2, 2014

DOI: 10.3892/ijmm.2014.1723

Abstract. Epithelial-mesenchymal transition (EMT) of lens epithelial cells (LECs) is the major pathological mechanism in anterior subcapsular cataract (ASC) and posterior capsule opacification (PCO), which are important causes of visual impairment. Extracellular signal-regulated kinase (ERK)1/2 pathway has been reported to play a major role in carcinogenesis, cancer metastasis and various fibrotic diseases. We hypothesized that ERK1/2 signaling can cross-interact with canonical transforming growth factor β (TGFβ)/Smad signaling and the Notch pathway, which subsequently contributes to LECs EMT. In this study, we demonstrated that ERK1/2 signaling was activated in TGFβ2-induced EMT in human LECs, whereas the blockade of TGFβ2/Smad2/3 signaling with SB431542 did not inhibit the activation of ERK1/2 induced by TGFβ2. In addition, inactivation of ERK1/2 signaling with a specific MEK/ERK1/2 inhibitor, U0126, completely prevented the TGFβ2-induced upregulation of α-SMA, collagen type I, collagen type IV and fibronectin. We also demonstrated that inactivation of ERK1/2 signaling inhibited canonical TGFβ/Smad signaling, as well as the Jagged/Notch pathway. By contrast, blockade of the Notch pathway by DAPT inhibited the TGFβ2-induced activation of ERK1/2 pathway in LECs. Thus, results of this study provide evidence for the complex interplay between ERK1/2, TGFβ/Smad, and Jagged/Notch signaling pathways in the regulation of EMT in LECs. Inhibition of the ERK1/2 pathway may therefore have therapeutic value in the prevention and treatment of ASC and PCO.

Introduction

Cataract is the most common cause of visual impairment in the elderly worldwide, particularly in developing countries (1). Anterior subcapsular cataract (ASC) and posterior capsule opacification (PCO) are different types of cataract that share similar cellular and molecular features (2,3). PCO, also known as a secondary cataract, is the most common long-term complication of modern cataract surgery. In the past few decades, although advances in surgical techniques, intraocular lens materials and designs have reduced the PCO rate, the incidence of PCO is still ~20-40% in adults and 100% in children (4,5). At present, cataract surgery and Nd:YAG laser capsulotomy are the only effective treatments for ASC and PCO, however, they are likely to induce many other complications and risks. Therefore, a better understanding of the pathogenesis of these diseases is critical for the development of new pharmacologic treatments.

Accumulating evidence has shown that the epithelial-mesenchymal transition (EMT) of lens epithelial cells (LECs) is a key pathological mechanism involved in the development of ASC (6,7) and PCO (8,9). PCO is caused by a wound healing response of residual LECs following cataract surgery. After surgery, the levels of various cytokines and growth factors increase in the aqueous humor and stimulate the residual LECs to proliferate and undergo EMT. Transforming growth factor β (TGFβ), especially TGFβ2, the major isoform in the aqueous humor of the eye, plays a central role in the cell biology of PCO (11). During the process of EMT, LECs undergo cytoskeletal rearrangement and loss of epithelial phenotype, then migrate away from the original location onto the posterior capsule, with the addition of a large amount of extracellular matrix proteins (collagen and fibronectin) deposition, and finally contribute to the development of PCO (7,10). Unlike PCO, ASC is a primary cataract that is mainly caused by ocular trauma, inflammation or irritation (12). The proliferation and EMT of LECs in situ lead to the formation of subcapsular plaques just beneath the lens anterior capsule, similar to the transdifferentiated cells in PCO (2). Thus, inhibition of the
proliferation of LECs and EMT may be a promising strategy to prevent ASC and PCO.

Several signaling pathways are involved in the process of LECs EMT in ASC and PCO development. Among these, canonical TGFβ/Smad signaling has been identified to occupy a crucial position in the signaling networks that control EMT of LECs. TGFβ/Smad signaling transmits signals by binding to the related transmembrane type I and II receptors, which subsequently phosphorylate receptor-regulated Smad2 and Smad3 (13). The phosphorylated Smad2/3 bind to the common mediator Smad4 to form a stable hetero-oligomeric complex, and then the complex translocates to the nucleus where the target gene expression is regulated (13). Recent studies have demonstrated that the blockade of TGFβ2/Smad2/3 efficiently prevents the effect of TGFβ2 on LECs migration, extracellular matrix production and EMT (14,15). In addition to the canonical Smad signaling, extracellular signal-regulated kinase (ERK) signaling is involved in TGFβ-induced EMT in different types of cells (16-19). The activation of ERK1/2 signaling enhances TGFβ-induced EMT, accompanied by morphological changes, the upregulation of EMT markers and extracellular matrix components. Blocking the function of ERK1/2 using a special inhibitor results in the inhibition of TGFβ-induced EMT (17,20). In LECs EMT, it has been previously reported that ERK1/2 is rapidly activated by TGFβ, and the specific inhibitor of ERK1/2 blocks the morphologic change of LECs and the upregulation of Slug induced by TGFβ (19).

Although the role of ERK1/2 signaling in EMT during cancer progression and some fibrotic disorders has been studied, the interaction of ERK1/2 with the canonical TGFβ/Smad signaling pathway and other signaling pathways in fibrotic diseases is poorly understood. In this study, we demonstrated that the TGFβ2-induced activation of ERK1/2 is independent of TGFβ/Smad signaling in human LECs, while the blockade of ERK1/2 signaling with the inhibitor U0126 completely prevents TGFβ2-induced EMT. Moreover, blockade of ERK1/2 signaling inhibits the canonical Smad signaling pathway, as well as the Jagged/Notch pathway. We also found that non-canonical TGFβ/ERK1/2 signaling can also be mediated by the Notch pathway. Taken together, these results suggested that ERK1/2 signaling cross-interacts with the TGFβ/Smad and the Jagged/Notch signaling pathways, thus mediating EMT in LECs.

Materials and methods

Reagents and antibodies. U0126 (a selective inhibitor of MEK1 and MEK2) and recombinant human TGFβ2 were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). SB431542 (a specific inhibitor for TGFβ receptor type I/ALK5 kinase that phosphorylates Smad2/3) and DAPT (an inhibitor of Notch receptor cleavage) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against ERK1/2, p-ERK1/2, Jagged-1, Notch-1, Notch-2, p-Smad2, p-Smad3, goat anti-rabbit and horse anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Cell Signaling Technology Inc. Antibodies against β-actin, α-SMA, collagen type I (Col I), collagen type IV (Col IV), and fibronectin (FN) were purchased from Abcam (Cambridge, UK).

Cell culture and treatment. The SRA01/04 human LEC line was kindly provided by Professor Fu Shang at the Laboratory for Nutrition and Vision Research (Boston, MA, USA), and cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS). The cells were grown at 37°C in a humidified atmosphere containing 5% CO2 and dissociated with 0.25% trypsin-0.02% ethylenediaminetetraacetic acid (EDTA) solution.

For TGFβ2 and U0126 treatments, the cells were seeded in 6-well plates and treated with 10 ng/ml recombinant human TGFβ2 and different concentrations of U0126 for different time-points.

Quantitative PCR analysis for gene expression. Total RNA was isolated from LECs using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and the RNA was then treated with DNase I (Sigma-Aldrich) to remove genomic DNA contamination. The concentration of total RNA was quantified by spectrophotometry and cDNA was synthesized with a reverse transcriptase kit (Takara Bio Inc., Otsu, Japan). For quantitative analysis of mRNA expression, the SYBR PrimeScript RT-PCR kit (Takara Bio Inc.) was used to amplify the target genes, and the reactions were performed with the ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s protocol. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control.

Western blot analysis for protein expression. The cells were washed twice with PBS, and then lysed in 100 µl of RIPA buffer with protease inhibitor cocktail for total protein extraction. Protein was collected after centrifugation and mixed with 5X SDS sample buffer. The samples were separated by 10% SDS-PAGE, and then transferred to PVDF membranes. The membranes were blocked with 5% non-fat milk for 1 h and the membranes were subsequently incubated with different primary antibodies at 4°C overnight. The membranes were washed with 1X PBS containing 0.1% Tween-20 (PBST) three times, and incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. The protein bands were detected with chemiluminescence detection reagents. β-actin was used as the loading control. Densitometric analysis was conducted by ImageJ software 1.41 (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. Experiments presented in the figures are representative of three or more different repetitions. Data were presented as mean ± standard error of the mean (SEM) and analyzed with SPSS 15.0 software (SPSS, Inc., Chicago, IL, USA). A standard Student’s t-test was used for statistical analysis. P<0.05 was considered to indicate statistical significance.

Results

Blockade of ERK1/2 signaling by U0126 prevents TGFβ2-induced EMT in LECs. To examine whether the blockade of ERK1/2 signaling prevented TGFβ2-induced EMT in LECs, U0126 (a selective inhibitor of MEK1 and MEK2) was used. EMT markers such as α-SMA, Col I, Col IV and FN
were investigated at mRNA and protein levels by quantitative PCR and western blot analysis, respectively. As shown in Fig. 1, quantitative PCR results showed that the mRNA expression of α-SMA, Col1, Col IV and FN were upregulated ~5.5- 4.3- 7.2- and 17.7-fold in TGFβ2-induced LECs for 24 h. In addition, western blot analysis results showed that TGFβ2 significantly increased the protein expression of α-SMA, collagen type I (Col I), collagen type IV (Col IV) and fibronectin (FN) in LECs (Fig. 2). Co-treatment with U0126 markedly abrogated the upregulation of α-SMA, Col I, Col IV and FN induced by TGFβ2 at the mRNA and protein levels (Figs. 1 and 2: P<0.05 vs. TGFβ2 treated with DMSO group). Maximum effect of U0126 was observed at a concentration of 20.0 µM, however, there was no obvious difference between 10.0 and 20.0 µM at mRNA level. These data suggested that the blockade of ERK1/2 pathway by U0126 effectively attenuated TGFβ2-induced EMT in LECs.

TGFβ2-induced ERK1/2 activation is independent of the canonical TGFβ/Smad pathway. To determine whether the canonical TGFβ/Smad signaling is required for the activation of ERK1/2 pathway by TGFβ2, SB431542 (a specific inhibitor for TGFβ receptor type I/ALK5 kinase that phosphorylates Smad2/3) was used. As shown in Fig. 3A and B, when LECs were stimulated by TGFβ2 for 30 min, ERK1/2 was activated via phosphorylation but with an unchanged total protein level, while U0126 treatment completely inhibited the TGFβ2-induced activation of ERK1/2. However, SB431542 treatment had no effect on the phosphorylation of ERK1/2 (Fig. 3A and B: P<0.05 vs. TGFβ2 treated with DMSO group). These results indicated that TGFβ2-induced ERK1/2 activation is independent of the canonical TGFβ/Smad pathway in LECs.
ERK1/2 PATHWAY MEDIATES EMT

CHEN et al: ERK1/2 PATHWAY MEDIATES EMT

U0126 mediates canonical TGFβ/Smad signaling by inhibiting the phosphorylation of Smad2. To examine whether there is a crosstalk between the ERK1/2 signaling and the canonical TGFβ/Smad pathway, the effect of U0126 on the activation of receptor-regulated Smad proteins Smad2 and Smad3 was examined. As shown in Fig. 3C and D, TGFβ2 alone clearly induced apparent phosphorylation of Smad2 and Smad3 following 60-min treatment, whereas co-treatment with U0126 inhibited the phosphorylation of Smad2, but had no effect on the phosphorylation of Smad3 in LECs (Fig. 3C and D: P<0.05 vs. TGFβ2 treated with DMSO group). Collectively, these data suggested that U0126 inhibits the canonical TGFβ2/Smad signaling transduction by inhibiting the phosphorylation of Smad2. Thus, there is a crosstalk between ERK1/2 signaling and the canonical TGFβ2/Smad signaling pathway in LECs.

U0126 prevents TGFβ2-induced EMT partly by inhibiting the Jagged/Notch pathway. Accumulating evidence suggests that the Notch signaling pathway is a vital regulator in the induction of EMT during embryonic development, cancer metastasis and various fibrotic diseases (21). Results of a previous study also found that the Jagged/Notch pathway is activated through canonical TGFβ2/Smad signaling during EMT in human LECs, while blockade of the Notch pathway with the specific inhibitor DAPT strongly inhibited TGFβ2-induced EMT (unpublished data). Therefore, we investigated whether inactivation of ERK1/2 signaling with U0126 inhibited Notch signaling activated by TGFβ2, and subsequently inhibited LECs EMT. As shown in Figs. 4 and 5, TGFβ2 treatment alone significantly increased the expression of Jagged-1, Notch-1 and Notch-2 at mRNA and protein levels, while U0126 completely attenuated the TGFβ2-induced upregulation of Jagged-1, Notch-1 and Notch-2 (Figs. 4 and 5: P<0.05 vs. TGFβ2 treated with DMSO group). In addition, U0126 treatment attenuated TGFβ2-induced Notch target genes Hes-1 and Hey-1 expression (Fig. 4: P<0.05 vs. TGFβ2 treated with DMSO group). These results suggested that U0126 prevents TGFβ2-induced EMT partly by down-regulating the Jagged/Notch pathway. Thus, non-canonical ERK1/2 signaling also contributes to the TGFβ2-induced activation of the Notch pathway in LECs.

Non-canonical TGFβ/ERK1/2 signaling can be mediated by the Notch pathway. It is unclear whether blockade of Notch signaling is able to modulate ERK1/2 signaling pathway activated by TGFβ2. As expected, blockade of the Notch pathway by DAPT clearly inhibited the TGFβ2-induced activation of ERK1/2 pathway in LECs (Fig. 6: P<0.05 vs. TGFβ2 treated with DMSO group). These results suggested that the non-canonical TGFβ/ERK1/2 signaling can be mediated by the Notch pathway conversely in LECs. This finding also indicated that there is a crosstalk between the ERK1/2 signaling and Notch pathways.
Discussion

A growing number of studies have proven that the development of ASC and PCO largely attributes to the EMT of LECs in response to a variety of cytokines, typically TGFβ2. Activation of ERK1/2 pathway plays a critical role in carcinogenesis, cancer metastasis, and various fibrotic diseases, including PCO (19,22-24). In this study, we investigated the role of ERK1/2 signaling in TGFβ2-induced EMT in human LECs, with a focus on the interaction of ERK1/2 signaling with the canonical TGFβ2/Smad and the Jagged/Notch pathways.

We found that the activation of ERK1/2 signaling by TGFβ2 stimulation is independent of the canonical TGFβ2/Smad pathway in LECs. In addition, inactivation of ERK1/2 strongly prevents the upregulation of EMT markers induced by TGFβ2. These results suggest that ERK1/2 signaling cross-interacts with the canonical TGFβ2/Smad and Jagged/Notch signaling pathways, thus regulating EMT in LECs.

TGFβ signaling occupies a key position in the signaling networks that regulates EMT. It includes canonical Smad signaling and non-canonical Smad independent signaling pathways. Previous studies have reported that ERK1/2 signaling is involved in TGFβ2-induced EMT in LECs and other types of cells (16-19). The activation of ERK1/2 signaling promotes TGFβ2-induced EMT and ECM components deposition, whereas the inactivation of ERK1/2 inhibits TGFβ2-induced EMT effectively (19,20). In the present study, we found that ERK1/2 is rapidly activated by TGFβ2 stimulation, and that MEK1/2 inhibitor U0126 blocks this response completely. Nevertheless, SB431542, a specific inhibitor for the canonical TGFβ2/Smad2/3 signaling transduction, has no effect on the activation of ERK1/2 induced by TGFβ2. These data indicate that TGFβ2-induced ERK1/2 activation is independent of the TGFβ2/Smad pathway in LECs. In addition, inactivation of ERK1/2 signaling strongly prevents the upregulation of EMT markers induced by TGFβ2. These results suggest that ERK1/2
signaling pathway is a critical mediator for TGFβ induction of EMT in LECs, and ERK1/2 inhibitor can be useful for abrogating EMT phenotype.

It has been reported that non-canonical Smad signaling, such as the p38MAPK and PI3K/AKT pathways, can cross-talk and integrate with the canonical TGFβ/Smad signaling, thereby contributing to EMT (25). To examine whether there is a crosstalk between the non-canonical TGFβ/ERK1/2 signaling and the canonical TGFβ/Smad signaling, the effect of U0126 on the activation of receptor-regulated Smad2 and Smad3 induced by TGFβ2 was investigated. We found that U0126 inhibits the phosphorylation of Smad2 induced by TGFβ2, but cannot inhibit the phosphorylation of Smad3 in LECs. These results suggest that U0126 mediates the canonical TGFβ/Smad signaling by inhibiting the phosphorylation of Smad2. Therefore, there is a crosstalk between the non-canonical TGFβ/ERK1/2 and the canonical TGFβ/Smad signaling in LECs EMT.

Evidence suggests that the Notch signaling pathway is a vital regulator in the induction of EMT during embryonic development, cancer metastasis and various fibrotic diseases (21). Activated Jagged/Notch signaling has been confirmed in a large range of fibrotic diseases developed in the kidney, liver and lung (26). Moreover, our former study found that the Notch signaling pathway is upregulated via canonical TGFβ2/Smad signaling in LECs EMT, while blockade of the Notch pathway with DAPT markedly reverses TGFβ2-induced EMT. In this study, we have shown that U0126 attenuates the TGFβ2-induced upregulation of Jagged-1, Notch-1 and Notch-2, as well as TGFβ2-induced Notch target genes Hes-1 and Hey-1 expression. These results suggest that non-canonical ERK1/2 signaling also contributes to the TGFβ2-induced activation of the Notch pathway in LECs. Inactivation of ERK1/2 with U0126 abrogates TGFβ2-induced EMT partly by suppressing the Jagged/Notch pathway. Furthermore, we observed that blockade of the Notch pathway by DAPT inhibits the TGFβ2-induced activation of the ERK1/2 pathway. This means non-canonical TGFβ2/ERK1/2 signaling can be mediated by the Notch pathway inversely in LECs. Collectively, these data indicate that there is a crosstalk between the ERK1/2 signaling and the Notch pathway in LECs EMT.

In summary, our results provide evidence that the TGFβ2-induced activation of ERK1/2 is independent of canonical TGFβ2/Smad signaling in human LECs. Inactivation of ERK1/2 signaling with U0126 completely inhibits TGFβ2-induced EMT in LECs. In addition, the blockade of ERK1/2 signaling inhibits the canonical Smad signaling pathway, as well as the Jagged/Notch pathway. We also found that non-canonical TGFβ2/ERK1/2 signaling can be mediated by the Notch pathway conversely. Thus, findings of this study suggest that ERK1/2 signaling cross-interacts with the canonical TGFβ2/Smad and the Jagged/Notch signaling path-
ways, thus mediating EMT in LECs. Therefore, ERK inhibitor may have therapeutic value in the prevention and treatment of ASC and PCO.

Acknowledgements

We would like to thank Professor Fu Shang for kindly providing the SRA01/04 human LEC line for this study. The study was funded by the grant from the Guangdong Natural Science Foundation (S2012020010878).

References