·Basic Research·

Mechanisms of inhibition of elemene on human lens epithelial cell proliferation *in vitro*

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Abstract

• AIM: To study the effects of elemene (Ele) on proliferation and cell cycle of human lens epithelial cells B3 (HLE-B3) and the mechanisms of its signal transduction.

• METHODS: Recombinant human basic fibroblast growth factor (rhbFGF) was used to induce proliferation of HLE-B3 cells, which were incubated with 80mg/L Ele for 24 hours. The inhibitory effects of Ele on the proliferation of HLE-B3 cells were evaluated by MTT method. The effect of Ele on HLE-B3 cell cycle was analyzed by flow cytometry (FCM). The expressions of protein kinase A (PKA) and protein kinase G (PKG) of HLE-B3 were also analyzed by FCM.

• RESULTS: Ele altered the cell cycle of HLE-B3 and effectively inhibited HLE-B3 cell proliferation induced by rhbFGF. Ele up-regulated PKA and down-regulated the expression of PKG in HLE-B3 cell.

• CONCLUSION: Ele inhibits HLE-B3 proliferation, making it an attractive potential agent in regimens to treat after-cataracts.

• KEYWORDS: lens epithelial cell; cell culture; after-cataract; elemene

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INTRODUCTION

After-cataract, also known as posterior capsular opacification (PCO), is one of the most common complications that decrease vision after extracapsular cataract extraction (ECCE). The average incidence of after-cataract is 30%-50% three years following cataract surgery, the incidence of after-cataract is 22%-26% in patients 65-80 years of age, and the incidence of after-cataract is 100% in infants [1]. After-cataract is induced by proliferating remnant lens epithelial cells (LEC) and their migration to the posterior capsule after cataract surgery. If we could effectively inhibit LEC proliferation, it is likely that we could prevent the formation of after-cataracts. Elemene (Ele), a main component of the Chinese herb *Rhizoma Curcuma*, has been shown to reduce thrombosis, reduce tumor cell proliferation, as well as induce tumor cell apoptosis [2,3]. Ele is currently used in clinic to treat gastrointestinal tumors and lung cancer [4,5]. We have previously shown that in bovine LECs, Ele decreases the expression of proliferating cell nuclear antigens, increases concentrations of intracellular-free Ca^{2+} and cAMP levels, reduces cGMP levels, and inhibits cell proliferation [6,7]. The cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA or A-kinase) is a tetramer of two regulatory (RI and RII) and two catalytic (C) subunits. It dissociates and releases active C subunits after cAMP binds to the R subunits. Two types of A-kinases differ in subcellular localization: type I A-kinase containing RI is predominantly cytoplasmic, whereas type II A-kinase containing RII is located in intracellular sites through its association with A-kinase-anchoring proteins [8-11]. PKA is an important kinase in intracellular signal transduction pathways because it regulates cell differentiation and proliferation as well as cell cycles. Protein kinase G (PKG) is a cGMP-dependent protein kinase. Increased level of intracellular cGMP and PKG accelerates the synthesis of nucleic acids and histones, and raises the level of cell proliferation to promote cell division and proliferation. In this study, our overall objective was to determine the mechanisms by which Ele inhibits proliferation and alters cell cycle progression in HLE-B3

cells. In our study, we used flow cytomerty (FCM) to determine PKA and PKG expression in HLE-B3 cells exposed to Ele as a means to understand how these two signal transduction pathways involve in cell proliferation.

MATERIALS AND METHODS

Materials All reagents were purchased from Sigma unless otherwise stated. Purified Ele (99.0%) was generously provided by Dalian Jinkong Pharmaceutical Co., Ltd (China). Recombinant human basic fibroblast growth factor (rhbFGF) was obtained from PeproTech Inc. (UK). Antibodies of PKA and PKG were purchased from Santa Cruz Biotechnology, Inc. (US). Goat anti-rabbit IgG labeled with FITC was purchased from Sino-American Biotechnology Co. (China).Frozen HLE-B3 cell suspensions were thawed at 37℃, and diluted with DMEM and centrifuged at 1000*g* for 5 minutes. The supernatant was removed and cells were washed 3 times in DMEM. Cell suspension of 5×10^9 cells/L were transfered to culture flasks and stored in a 37℃ 50mL/L CO_2 humidified incubator.

Methods

HLE–B3 proliferation detection HLE-B3 suspensions (5×10^9 cells/L) were incubated for 24 hours in 96-well tissue culture plates (100μL per well) with DMEM supplemented with 25mL/L newborn calf sera (NCS) to synchronize cell cycles. Three experiment groups were: (1) rhbFGF group (10μg/L rhbFGF), (2) Ele group (whit 10μg/L rhbFGF and 80 mg/L Ele), and (3) control group (DMEM only). After 24 hours in culture and removal of the supernatant, experimental groups 1 and 2 were washed with PBS buffer solution containing 100μL MTT (0.5g/L), incubated in the dark at 37℃ for 4 hours, and treated with 100μL DMSO per well with gentle shaking. Absorbance value was measured by Microplate Reader at 490nm wavelength. Rates of cell proliferation suppression in the Ele group 2 were determined by the formula: Suppression rate (%)=(the average *A* of rhbFGF group-the average *A* of Ele group) ×100%/the average *A* of rhbFGF group.

Cell cycle analysis Approximately $(1\text{-}2)\times10^9$/L HLE-B3 cells were incubated with 80mg/L of Ele for 24 hours, washed with 0.01mol/L PBS (pH 7.2), pentions to fix cells at 4℃ for 12 hours. Cells were washed twice with cold PBS and centrifuged at 1000g for 5 minutes. Subsequently, cell pellets were resuspended in 200μL binding buffer with 30μL RNase A, and then incubated for 15 minutes in the dark at room temperature (RT). Finally, cells were analyzed by flow cytometry after adding 300μL binding buffer with 5μL propidium iodide (PI) (50mg/L).

PKA and PKG expression in HLE–B3 cells Approximately $(1\text{-}2)\times10^9$/L HLE-B3 cells were incubated with 80mg/L Ele for 24 hours, fixed with 1mL cold 1000mL/L methanol, stored at -20℃ for at least 10 minutes, centrifuged, and supernatant-removed. Cells were then washed with PBS without Ca^{2+} and Mg^{2+}, centrifuged, and supernatant-removed. The cells were resuspended in 50μL PBS with 10g/L BSA and 10mL/L bovine serum, mixed and placed at room temperature for 30 minutes. The suspensions were mixed with 50μL of 1:100 solution (0.01mol/L of sodium phosphate buffer at pH 7.6) of rabbit anti-human PKA and PKG polyclonal antibody and placed on ice for 30 minutes. For the negative control cells (Group 3), the procedure was identical except cells were incubated with PBS instead of PKG polyclonal antibody. Cells were then washed twice with PBS, incubated with 50μL of goat anti-rabbit IgG-FITC, and placed on ice in the dark for 30 minutes. Finally, cells were washed twice with PBS, and analyzed by a flow cytometer for levels of PKA and PKG.

Statistical Analysis The data were presented as the mean± SD. Differences were evaluated by using one-way ANOVA test. The level of statistical significance was $P<0.05$. Statistical analyses were performed by SPSS version 12.0.

RESULTS

Effect of Ele HLE–B3 The *A* of HLE-B3 cells in rhbFGF group (0.599±0.053) was significantly higher than that of the control group (0.409±0.042), $P<0.01$, $n=8$. This showed that activity of HLE-B3 cells in the rhbFGF group had markedly increased. The *A* of HLE-B3 cells in the Ele group (0.450±0.061) was significantly lower than that of the rhbFGF group ($P<0.01$, $n=8$), indicating that the activity of HLE-B3 cells had significantly decreased with Ele, and that Ele had effectively inhibited the proliferation of HLE-B3 cells induced by rhbFGF. The inhibition rate was 24.9%.

Effect of Ele on HLE–B3 Cell Cycle The number of cells in the G1 phase in the rhbFGF group had decreased significantly compared with the control group (42.1%±1.3% *vs* 46.4%±3.8%, $P<0.05$, $n=6$). Compared with the rhbFGF group, cells in the G1 phase in the Ele group (60.7%±2.1%) had significantly increased($P<0.01$); however, cells in the S phase of the rhbFGF group had increased significantly compared with the control group (51.6%±1.1% *vs* 31.8%±2.8%, $P<0.01$).Cells in the S phase in the Ele group (30.2%±3.4%) had significantly decreased compared with those in the rhbFGF group ($P<0.01$). Cells in the G2 phase in the rhbFGF group were significantly decreased compared with controls (6.2%±1.0% *vs* 21.7%±3.8%, $P<0.01$). The cells

in the G2 phase of the Ele group had increased (9.1% ± 1.7%) significantly compared with the rhbFGF group (P< 0.01). These results demonstrated that Ele altered the cell cycle of HLE-B3 and effectively inhibited rhbFGF-induced HLE-B3 cell proliferation.

Effects of Ele on PKA and PKG Expression The protein expression of PKA in HLE-B3 cells in the rhbFGF group (33.2%±2.2%) had decreased significantly compared with those in the control group (46.8%±1.7%), P<0.01, n= 6; protein expressions of PKA in HLE-B3 cells from the Ele group (64.1%±2.9%) was significantly higher than those of rhbFGF group (P<0.01, n=6). These findings indicate that rhbFGF down-regulated PKA expression in HLE-B3 cells, whereas Ele increased PKA expression in the cells. The protein expression of PKG in HLE-B3 of rhbFGF group (86.6% ±1.3%) was significantly higher than the control group (63.8%±0.5%), P<0.01, n=6 while expression in Ele group (55.1%±2.1%) was significantly less than the rhbFGF group (P <0.01, n =6). This showed that rhbFGF was increased; and Ele was decreased; the expression of PKG in HLE-B3 cells.

DISCUSSION

Previous study from Jie *et al* [10] demonstrated that Ele suppresses cell proliferation by inhibiting DNA synthesis induced by vascular endothelial growth factor in bovine retinal pigment epithelial cells. Their results showed that the *in vitro* anti-proliferative activity of beta-elemene mono-substituted amine and Re (CO) (3)-beta-elemene derivatives in human cervix epitheloid carcinoma HeLa cells were increased significantly compared with the derivative and parent beta-elemene. These derivatives reduced reinoblastoma protein (Rb) phosphorylation and cyclin D1 protein expression to arrest the cell cycle at G1 phase. In our study, we found that rhbFGF significantly reduced both G1 and G2 phases of HLE-B3 cells, whereas cells in S phase had increased significantly. On the other hand, after Ele reduced the proliferation of HLE-B3 cells, cell G1 and G2 phases had increased and S phase cycle had decreased. Thus, Ele likely inhibits the systhesis of HLE-B3 DNA, which prevents the cell transformation from G1 to S phase and slows cell cycle progression, further suppressing HLE-B3 cell proliferation. Protein kinase A (PKA), known as cyclic AMP-dependent protein kinase A, transfers the ATP phosphate group to the residues of serine or threonine of a specific protein for phosphorylation. It is believed that nearly all cAMP functions in eukaryotic cells are activated by PKA, enabling substrate protein phosphorlation. PKA, an important kinase in intracellular signal transduction pathway, regulates cell differentiation and proliferation, and plays an important role in adjustment of cell cycle. The PKA system is a signal transduction pathway in the G protein-coupled system. Extracellular signaling molecules bind to cell-surface receptors, and activate adenylate cyclase by G proteins, which in turn produce a second messenger of cAMP that activates PKA to amplify signals. This pathway is known as the PKA signal transduction system. Tang *et al* [12] found that compared with controls, acetylcholine significantly increased the cAMP levels and PKA activity in gastric cancer cells, further inhibiting cancer cell proliferation and differentiation. Further, Liu *et al* [13] found that inhibiting PKA activity enabled the expression of the apotosis regulator protein bcl-2, inducing apoptosis and inhibiting proliferation in T24 bladder cancer cell lines, further increasing their sensitivity to chemotherapeutic agents. Here, we found that 24 hours after rhbFGF acted on HLE-B3 cells, PKA expression decreased significantly, whereas Ele increased the PKA expression in the cells. This showed that PKA activation is an important aspect in the Ele-induced proliferation of HLE-B3 cells. Ele activated PKA activity in HLE-B3, enabling the phosporylation of certain serine and threonine residues in target proteins necessary for HLE-B3 division and proliferation, inhibiting cell cycle progression. The results of this study also indicated that the PKA signal transduction pathway plays an important role in Ele-induced inhibition of HLE-B3 proliferation. Our previous experiments demonstrated that Ele increasd cAMP levels in LEC [6,7]. We observed that agents that elevate intracellular cAMP levels (*e.g.*, elemene, curcumin, and triptolide) inhibited cell proliferation, a response also associated with an increased PKA activity. Our future studies will focus on inhibitors of PKA, (*e.g.*, KT5720, Rp-cAMPS, and 4-cyano-3 methylisoquinoline) and how they block PKA-mediated HLE-B3 proliferation.

PKG is a cGMP-dependent protein kinase, which consists of two polypeptide chains of serine and threonine protein kinases. PKG has several characteristics. The compound (1) binds with cGMP to become active; (2) catalyzes the phosphorylation of hydroxyl groups of serine and threonine, which requires a high proportion of basic amino acids around the phosphorylated serine or threonine; and (3) is different from PKA in enzyme activation, inhibition, and regulation. Increases in the level of intracellular cGMP and the activity of PKG will accelerate the synthesis of nucleic acids and histones, and raise the level of cell proliferation to promote cell division and proliferation. We found that after 24 hours of rhbFGF incubation, PKG expression in HLE-B3

cells had significantly increased (86.6%±1.3%); however, when Ele was incubated with HLE-B3 cells, the PKG express was significantly lower (55.1%±2.1%).This indicated that Ele decreased HLE-B3 PKG expression, inhibited the synthesis of cellular DNA and histone, reduced the level of cell proliferation, and thus suppressed HLE-B3 division and proliferation through the PKG pathway. However, further studies are needed to determine the precise roles of PKG and cGMP-PKG in HLE-B3 proliferation. Overall, our findings suggest that the increased PKA and decreased PKG expression in HLE-B3 is one major mechanism by which Ele inhibits cell proliferation, possibly by activating the PKA signaling pathway or interfering with PKG signal transduction. Future trials will be required to study Ele in the clinic with patients who develop after-cataracts.

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