• Basic Research •

Effect of dopamine on bone morphogenesis protein-2 expression in human retinal pigment epithelium

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Abstract

• AIM: To investigate the effect of dopamine on bone morphogenesis protein-2 (BMP-2) expression in retinal pigment epithelium (RPE) cells *in vitro*.

• METHODS: ARPE-19 cells as a human RPE cell line were cultured with dopamine for different times (2, 4, 6, 8, 12, 16 and 24h) or with different concentrations (0.1, 1, 2, 5, 10, 20, and 100 μg/mL) *in vitro*. BMP-2 mRNA expression level in ARPE-19 cells was analyzed with real-time polymerase chain reaction (PCR) analysis and BMP-2 protein level was measured with Western blot analysis. The active form of BMP-2 in the culture medium was measured with enzyme-linked immunosorbent assay (ELISA).

• RESULTS: The expression level of BMP-2 increased significantly cultured with 20 μg/mL dopamine, at different time points (*P*<0.05). BMP-2 mRNA level peaked 2h and the protein level peaked at 6 and 8h after treatment. The concentrations of secreted BMP-2 elevated at 12h and peaked at 24h (*P*<0.05) in a time-dependent manner. Treated with 100 μg/mL dopamine for 6h, the expression levels of BMP-2 mRNA and protein in ARPE-19 cells were enhanced significantly compared to that in the untreated cells (*P*<0.05). And secreted BMP-2 protein in the cell culture supernatant was also increased (*P*<0.05).

• CONCLUSION: Dopamine up-regulate BMP-2 expression in RPE cells, and this may be associated with its inhibitive effect on myopia development.

• KEYWORDS: dopamine; retinal pigmental epithelieum; bone morphogenetic protein-2

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INTRODUCTION

Dopamine is a chemical messenger for light adaptation, and it involves trophic functions of the retina, including growth, development, cell death and experimental myopia[1]. In animal models of myopia, dopamine receptor agonist and dopamine have inhibitory effect on form-deprived myopia (FDM)[2] and lens-induced myopia (LIM)[3]. So dopamine serves as a signaling molecular in regulating ocular growth[4], but its mechanism is unclear. Dopamine is released from the dopaminergic amacrine cells in the inner plexiform layer and the vast majority of retinal cells respond to dopamine[5]. All of the retinal cells bear dopamine receptors, including retinal pigment epithelium (RPE) cells[1]. Study has shown that dopamine pathways might be involved in the control of interphotoreceptor matrix properties and retina/RPE interaction[4,6].

In LIM and FDM, the retinal dopamine level decreases[4-5], but the choroidal and scleral dopamine levels are not changed[4]. So we proposed that dopaminergic mechanisms of regulating ocular growth may lie in modulating the secretion of growth factors by the RPE cells to relay the information from the retina to the sclera[7].

Bone morphogenesis protein-2 (BMP-2) is an important factor for early eye morphogenesis, ocular development and growth[8-9]. BMP-2 expression in chick retina/RPE is down-regulated in FDM[8]. BMP-2 and its receptors are found in human sclera fibroblasts and it influences extra cellular matrix synthesis *in vitro*[10-11]. The gene-analysis of humans confirmed that BMP-2 related to refractive error and myopia[12-13].

In the present study, we investigate the effect of dopamine on the level of BMP-2 expression in RPE cells *in vitro*.

MATERIALS AND METHODS

Cell Culture and Handling ARPE-19 (ATCC Number: CRL-2502) cells as a human RPE cell line were routinely cultured in a DMEM/F12 (1:1) medium containing 10% fetal bovine serum (FBS, Invitrogen Life Technologies, Carlsbad, CA, USA) and 100 IU/mL penicillin/streptomycin (Gibco, CA,

USA) at 37℃ in 5% (vol/vol) CO_2 humidified atmosphere. The medium was changed every 2-3d. Cells were routinely passaged at a confluence between 80% and 90% and at a split ratio ranging from 1:3 to 1:5 by digestion in 0.05% trypsi-0.02% ethylene diamine tetraacetic acid (Invitrogen Life Technologies, Carlsbad, CA, USA). Stock solution was prepared by dissolving dopamine (Sigma-Aldrich, St. Louis, MO, USA) in ddH_2O at a concentration of 100 mg/mL. ARPE-19 cells were cultured with 20 μg/mL dopamine for 2, 4, 6, 8, 12, 16 and 24h respectively, or with different concentration of dopamine (0.1, 1, 2, 5, 10, 20, 100 μg/mL) for 6h. The treament was repeated for 3 times respectively.

Real-time Polymerase Chain Reaction Total RNA was isolated from cells with Trizol reagent (Invitrogen Corp. Carlsbad, USA) according to the manufacturer's instructions. The cDNA was synthesized from 500 ng of total RNA (Takara Biotechnology, Japan). Real-time polymerase chain reaction (PCR) was then performed with Roche 4800 real-time-PCR system with SYBR Green I Master mix (Roche Diagnostics GmbH, Mannheim, Germany). The relative quantification of the target sequence and the reference sequence requires that the PCR efficiencies of the BMP-2 gene and the β-actin gene PCR reactions be close to the identical. During the log-linear phase, amplification can be described by N=No(1+E)n, where N is the number of amplified molecules, No is the initial number of molecules, E is the amplification efficiency, and n is the number of cycles. If the amplification efficiency is similar for the two reactions, the initial concentration of the sample is calculated on the basis of the above formula by using the comparative delta Ct method and the gene copy number was given by the formula $2^{-(\Delta\Delta Ct)}$. The sequences of the primer pairs were: BMP-2 [GenBank: NM_001200.2]: forward primer 5'-ACTCGAAATTCCCCGTGACC-3' and reverse primer 5'-CCACTTCCACCACGAATCCA-3'; β-actin [GenBank: NM_001101.3]: forward primer 5'-CCAGAGGCGTACAGGGATAG-3' and reverse primer 5'-CCAACCGCGAGAAGATGA-3'. The expected sizes for the BMP-2 and β-actin PCR products were 144 and 97 bp, respectively. After amplification, the products were melted and the melting curve of the two genes has only one peak respectively, so the primer has great specificity.

Western Blot Analysis The total protein of cultured cells was collected in lysis buffer with 1% phenylmethanesulfonyl fluoride (PMSF, all from Beyotime, Shanghai, China) and its concentration was assayed by the BCA protein assay kit (Beyotime, Shanghai, China). Totally 20 μg protein samples were separated by 12% SDS-PAGE and electrotransferred to PVDF membranes (Millipore, Billerica, MA) with 200 mA for 1h, which were blocked with 5% milk (Sigma Chemical Co. St. Louis, USA) for 2h at room temperature. Subsequently, the membranes were incubated with anti-β-actin antibody (ab8226, 1:3000 dilution; Abcam, San Francisco, USA), anti-BMP-2 antibody (ab6285, 1:500 dilution; Abcam, San Francisco, USA) overnight at 4℃, followed by the HRT-conjugated goat anti-mouse IgG antibodies (BA1051, 1:1000 dilution, Boster Biological Technology) antibody for 1h at room temperature. Proteins were detected with the enhanced chemiluminescence (ECL) detection system and exposed onto a negative film, developed, and fixed. The film was scanned and then analyzed with Bio-Rad Quautity Imaging software (version 1, Bio-Rad Laboratories). β-actin served as an internal reference, and the experiments were repeated at least three times.

Enzyme Linked Immunosorbent Assay Determination APRE-19 cells were plated at a concentration of 2×10^4 per well in 24 well plates containing DMEM plus 10% FBS and allowed to attach for 18h. Cells were washed with phosphate buffered solution (PBS) and re-fed with 0.5 mL of DMEM without FBS for 7h. Dopamine was added to cells at the different concentrations or for different times, and then the culture supernatants were collected to analyze the BMP-2 protein concentration using BMP-2 Quantikine enzyme linked immunosorbent assay (ELISA) Kit (DBP200, R&D System, MN, USA). Luciferase units obtained were normalized to the protein content of each well. All experiments were performed at least three times with four independent wells per condition.

Statistical Analysis Each experiment was repeated at least three times. Data were expressed as mean±standard error of the mean (SEM). The statistical significance of differences between cell groups *in vitro* was determined by one-way ANOVA followed by Tukey's post hoc multiple comparison tests using the Mann-Whitney *U* test when significance was detected. All the data analysis was performed using SPSS (version19.0, SPSS Inc., Chicago, USA). $P<0.05$ was regarded as statistically significant.

RESULTS

To investigate the effect of dopamine on BMP-2 expression in ARPE-19 cells *in vitro*, the levels of BMP-2 mRNA and protein expression in the ARPE-19 cells and the concentrations of BMP-2 production in the supernatants of ARPE-19 cells were measured. When ARPE-19 cells were cultured with 20 μg/mL dopamine, the BMP-2 mRNA and protein levels increased significantly at 2, 4, 6, 8, 12, 16 and 24h ($P<0.05$; Figure 1). BMP-2 mRNA expression level in treated cells peaked after 2h and it increased 4.0-fold (Figure 1A), and the protein level peaked after 6 and 8h and it elevated 2.1-fold (Figure 1B, 1C) when compared wtih the control. The concentrations of secreted BMP-2 had no obvious changes after 6 and 8h treatment but it elevated significantly at 16h and peaked at 24h increasing 2.0-fold ($P<0.05$; Figure 2). Our results indicated that the production of BMP-2 by ARPE-19 could be induced by dopamine in a time-dependent manner.

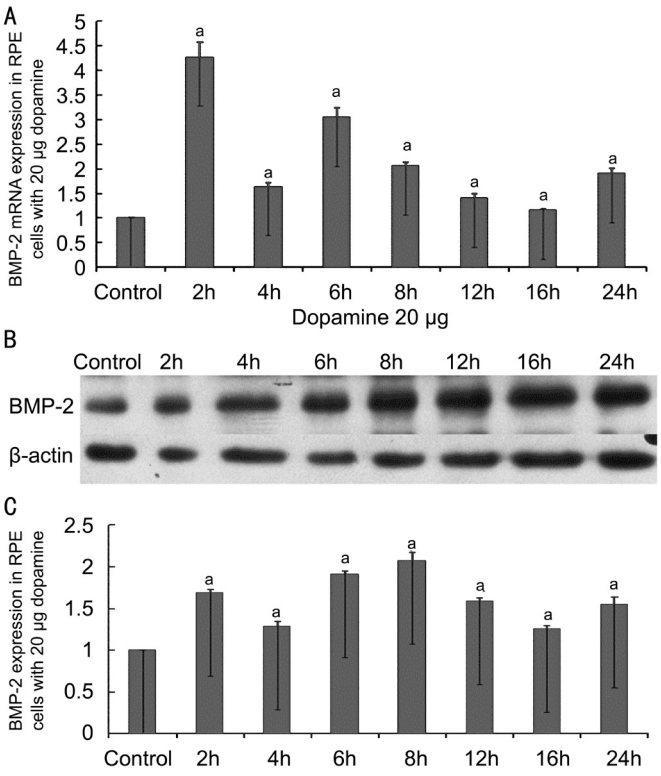

Figure 1 The effect of dopamine on BMP-2 expression in ARPE-19 cells for different times Dopamine (20 μg/mL) enhanced the expression of BMP-2 mRNA (A) and protein (B, C) in ARPE-19 cells. This effect existed after 2h treatment with dopamine and lasted after 24h. The data were means±SEM (n=3, ^aP<0.05 *vs* the control).

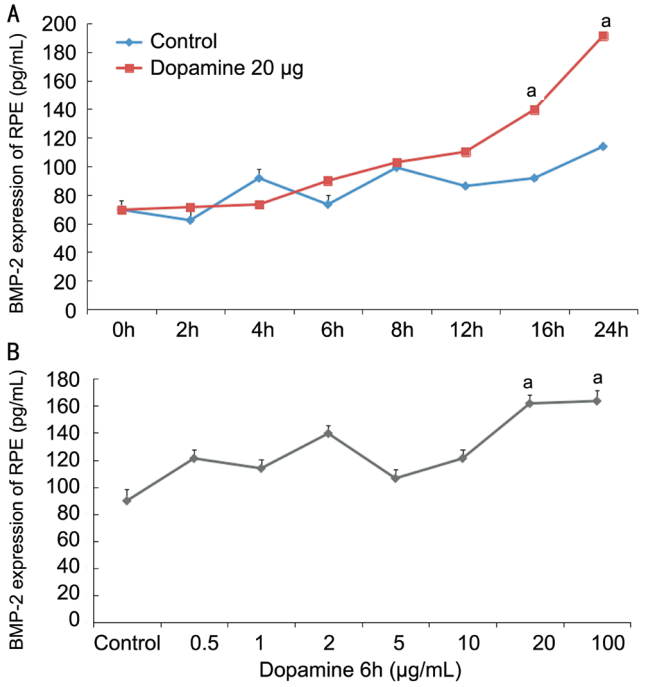

Figure 2 Dopamine stimulated secretion of BMP-2 in the supernatants of ARPE-19 cells BMP-2 protein in the media was measured by ELISA and normalized to cell counts (10^6). With 20 μg/mL dopamine in culture, the BMP-2 concentration increased significantly after 16 and 48h (A). There were no statistically significant differences when comparing the concentrations of secreted BMP-2 after 2, 4, 6, 8 and 12h. When ARPE-19 cells were treated with 20 and 100 μg/mL dopamine for 6h, the concentration of secreted BMP-2 increased significantly (B). The data were means±SEM (n=3, ^aP<0.05 *vs* the control).

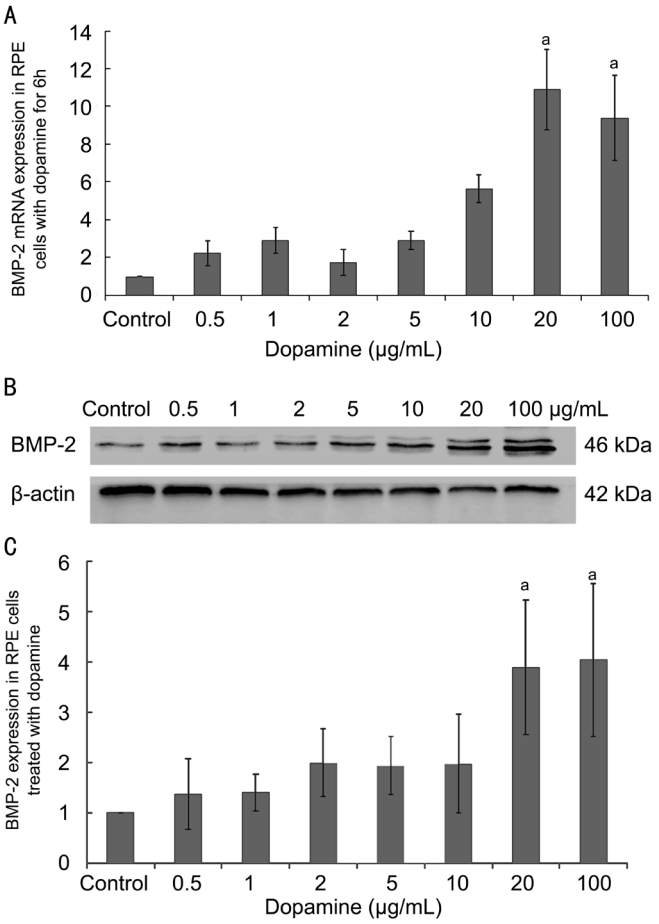

Figure 3 BMP-2 expression in ARPE-19 cells cultured with dopamine for 6h With 20 or 100 μg/mL dopamine in the culture, levels of BMP-2 mRNA (A) and protein (B, C) increased significantly. The data were means±SEM (n=3, ^aP<0.05 *vs* the control).

With 20 and 100 μg/mL dopamine in the culture for 6h, BMP-2 mRNA level increased 10.9-fold and 9.4-fold seperately (Figure 3A), and BMP-2 protein level increased nearly 4.0-fold compared to the untreated cells (Figure 3B, 3C). The active form of BMP-2 in the supernatant was also enhanced significantly with 20 and 100 μg/mL dopamine (Figure 2B). Between the two concentration of dopamine, their effect on BMP-2 expression and secration has no differences (P>0.05). There were no statistical differences in BMP-2 mRNA and protein expression levels , and the concentrations of secreted BMP-2 also had no differences when comparing the cells cultured with 0.5, 1, 2, 5 and 10 μg/mL dopamine with the control cells (P>0.05).

DISCUSSION

This study demonstrated that dopamine stimulated BMP-2 expression in RPE cells *in vitro* and this may be associated with the inhibitive effect of dopamine on ocular growth and myopia development.

Many studies have shown that dopamine pathways are involved in myopia development[14] and the inhibitory effects of dopamine on FDM and LIM are mediated through stimulation of the dopamine D1-receptor and D2-receptor,

especially the D2-receptor[15]. Apomorphine blocking myopia may be by a dopamine D2-receptor mechanism acting in retina or pigmented epithelium[5]. RPE plays a role in eye growth and refractive development by relaying signals between the retina and adjacent choroidal and scleral layers[16-17]. RPE can modulate fluid exchange between the retina and choroid inducing the choroidal thickness changes and scleral growth [17-19]. *In vitro* RPE cells directly influenced the scleral glycosi aminoglyca good (GAG) synthesis[19]. Therefore, we proposed that dopamine and its agonist influced the production and release of some growth factors from RPE cells, which normally affected the growth of scleral chondrocytes. In our study, dopamine increased the secretion of BMP-2 and the expression levels of BMP-2 mRNA and protein in RPE cells.

BMP-2 belongs to the BMPs family and its expression has been detected in both adult and embryonic tissues of cornea, trabecular meshwork, optic nerve head, retina and conjunctiva[20]. BMP-2 is involved in the pathophysiology of several ocular diseases and regulating myopia development[21]. During myopia development, choroidal BMP-2 gene expression involves in vascular regulation and/or angiogenesis and its expression alternation may be part of a common choroidal response[22]. *In vitro*, BMP-2 influences scleral extracellular matrix synthesis[11]. In relative terms, BMP-2 appears more highly expressed in RPE layer compared to retina and choroid[21], suggesting BMP-2 may be produced by RPE cells. In this study, we demonstrated that the secreted BMP-2 increased with dopamine, so it may reach retina, choroid and sclera by diffusion to affect their function.

In our study, we presented that dopamine stimulated secretion of BMP-2 by RPE cells *in vitro*. This suggested that dopamine blocking myopia development was possibly through modulation RPE secretory activity of growth factors such as BMP-2 into retina, choroid and sclera.

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Conflicts of Interest: Li HH, None; **Sun YL,** None; **Cui DM,** None; **Wu J,** None; **Zeng JW,** None.

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