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 pigment epithelium; bone morphogenetic protein-2

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[6]. 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 extracellular 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°C in 5% (vol/vol) CO₂ 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% trypsin-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 ddH₂O at a concentration of 100 mg/mL. ARPE-19 prepared by dissolving dopamine (Sigma-Aldrich, St. Louis, Life Technologies, Carlsbad, CA, USA). Stock solution was trypsi-0.02% ethylene diamine tetraacetic acid (Invitrogen passaged at a confluence between 80% and 90% and at a

The medium was changed every 2-3d. Cells were routinely

treated 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−(ΔΔCt). The sequences of the primer pairs were: BMP-2 [GenBank: NM_001200.2]: forward primer 5'-ACTGAAATTCCTCGTACCC-3' and reverse primer 5'-CCACTTCCACGAAATCCA-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 in ARPE-19 cells

Enzyme Linked Immunosorbent Assay Determination APRE-19 cells were plated at a concentration of 2×10⁵ 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.

Enzyme Linked Immunosorbent Assay Determination

APRE-19 cells were plated at a concentration of 2×10⁵ 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 (version 19.0, 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 with 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.
With 20 and 100 µg/mL dopamine in the culture for 6h, BMP-2 mRNA level increased 10.9-fold and 9.4-fold separately (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 secretion 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.

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 secretion 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).

**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, *P<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, *P<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, *P<0.05 vs the control).
extracellular matrix synthesis in the cornea, trabecular meshwork, optic nerve head, retina and choroid. BMP-2 belongs to the BMPs family and its expression levels of BMP-2 mRNA and protein in RPE cells. 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 the cornea, trabecular meshwork, optic nerve head, retina and conjunctiva. BMP-2 is involved in the pathophysiology of several ocular diseases and regulating myopia development. 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. In vitro, BMP-2 influences scleral extracellular matrix synthesis. In relative terms, BMP-2 appears more highly expressed in RPE layer compared to retina and choroid, 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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REFERENCES