Effect of adiponectin on expression of vascular endothelial growth factor and pigment epithelium-derived factor: An in vitro study

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ABSTRACT

Aim: This study was carried out to identify the role of adiponectin (APN) in modulating the expression of vascular endothelial growth factor (VEGF) and pigment epithelial-derived factor (PEDF) in relation to ocular angiogenesis.

Materials and Methods: Human retinal pigment epithelial cell lines (ARPE-19) were cultured in Dulbeco’s minimum essential medium with 10% fetal bovine serum (FBS) and exposed to varying concentrations of recombinant adiponectin (5–5 ng/ml) for 1 h. Analysis of VEGF and PEDF mRNA was done by reverse transcriptase and further quantified by quantitative polymerase chain reaction. VEGF and PEDF protein expression were studied using enzyme-linked immune sorbent assay (ELISA).

Statistical Analysis: Unpaired Student’s t-test was used to analyze the significance. P < 0.05 was accepted as statistically significant.

Results: ARPE cells exposed to APN showed decreased expression of VEGF mRNA, protein whereas PEDF protein is unaltered and PEDF mRNA was increased.

Conclusion: Our in vitro study on ARPE exposed to APN showed a negative correlation with VEGF levels. Thus indicating the protective role for APN in angiogenesis-related diseases.

KEY WORDS: Adiponectin, pigment epithelium derived factor, proliferative diabetic retinopathy, vascular endothelial growth factor

Introduction

Adiponectin (APN), a circulating adipokine exerts in oligomeric and globular forms which are regulators of energy homeostasis. APN is reported to have anti-diabetic, anti-inflammatory, anti-angiogenic, anti-atherogenic and anti-hypertensive properties.[1,2] APN is expressed in choroidal tissues, and it inhibits the LASER-induced choroidal neovascularization up to 78% by intraperitoneal injections in experimental mouse model.[3] Recently elevated levels of APN in aqueous humor[4] and in vitreous[5] of the patients with proliferative diabetic retinopathy (PDR) were reported. Expression and localization of APN and its receptor in retinal layers and in various ocular tissues including primary isolated ocular cell lines[6] indicates its role in ocular physiology and pathology.

Vascular endothelial growth factor (VEGF) is an important proangiogenic factor and is found to be elevated in vitreous of patient with ocular angiogenesis viz PDR.[7] pigment epithelial-derived factor (PEDF) is a known antiangiogenic factor and is found to be significantly lower in patients with PDR and proportional to ocular neovascularisation.[8] These two cytokines are the mostly studied molecules in relation to ocular angiogenesis. Retinal pigment epithelial (RPE) is a good model to study the alteration in these cytokines. It is a single layer of pigment epithelial cells,[9] which secretes both pro angiogenic VEGF anti angiogenic cytokine PEDF[10] and maintains the outer retinal barrier.

The primary aim for the current treatment for ocular angiogenesis is to inhibit the abnormal blood vessel formation by targeting VEGF. Although currently available anti-VEGF drugs to treat PDR namely bevacizumab, ranibizumab, pegatunib sodium are reported to be useful, but not without limitations.[11] Therefore, search on new targets for therapeutic application...
is an active field of research. The possible role of APN in the disease process is discussed from the results of our cell culture experiments.

**Materials and Methods**

**Cell Culture Experiment**

Human retinal pigment epithelial cell lines (ARPE-19) cells were purchased from American Type Culture Collection (ATCC). Cells were cultured and maintained in Dulbeco’s minimum essential medium (DMEM) medium with 10% fetal bovine serum (FBS) in the house. Trypan blue exclusion was done to check the cell viability. In the view of the fact that only higher doses of APN were studied, effect of APN at lower concentration were selected for our experiment. Human ARPE-19 cells were cultured in DMEM medium supplemented with 10% FBS at 37°C in a humidified 5% CO₂. The experiment was carried out in ARPE-19 cells by treating cells with varying concentrations of recombinant APN (rAPN) (R and D, USA) from 5 pg/ml, 50 pg/ml, 500 pg/ml, 5 ng/ml for 1 h after overnight starvation with serum free DMEM and all experiments were carried out in triplicates.

**Reverse Transcriptase and Quantitative Real-Time Quantitative Polymerase Chain Reaction**

RNA extraction was done from cell lines ARPE-19 by Tri method. 1-2 μg RNA was reverse transcribed by iscript cDNA synthesis kit (Biorad Laboratories Inc., USA) and the resulted cDNA was used as the template for amplification of PEDF[12] and VEGF.

**Polymerase Chain Reaction Conditions**

**Vascular endothelial growth factor**

Annealing temperature 60°C; thermal cycles –40 cycles; product size –180 bp.

**Pigment epithelial derived factor**

Annealing temperature 63°C; thermal cycles –40 cycles; product size –155 bp.

**Glyceraldehyde-3-phosphate dehydrogenase**

Annealing temperature 63°C; thermal cycles –30 cycles; product size –495 bp.

Reverse transcriptase and real time polymerase chain reaction (RT-PCR) were performed using the above mentioned primers [Table 1].

Real-time PCR was performed using SYBR green PCR master mix (Eurogentec, Europe) on an ABI 7300 instrument. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was run as an internal control for all the experiments. The values of specific genes were normalized to GAPDH. Quantitative PCR was carried out in triplicate.

**Quantification of vascular endothelial growth factor and pigment epithelial-derived factor**

vascular endothelial growth factor were measured using quantikine enzyme-linked immune sorbent assay (ELISA) kit (R and D, USA). PEDF was measured using chemikine PEDF ELISA kit (Chemicon International, USA). After exposure of varying concentration of rAPN, conditioned medium was collected and concentrated using speed vacuum for VEGF ELISA and the steps were followed as described by supplier.

**Statistical Analysis**

All values are expressed as mean ± SD Unpaired Student’s t-test was used to analyze the significance. P < 0.05 was accepted as statistically significant.

**Results**

**Adiponectin Exposure to Retinal Pigment Epithelial Decreased Vascular Endothelial Growth Factor Protein Expression**

Conditioned medium of cells after exposure of rAPN along with the control were used to quantify VEGF and PEDF protein expression. VEGF protein expression was decreased significantly in cells treated with 5.0 pg rAPN (P < 0.007), 50 pg rAPN (P < 0.01), 500 pg rAPN (P < 0.02), 5000 pg rAPN (P < 0.01) treated cells compared to control [Figure 1a] whereas PEDF protein didn’t show much variation nor statistically significant in cells treated with 5.0 pg rAPN (P < 0.46), 50 pg rAPN (P < 0.34), 500 pg rAPN (P < 0.18), 5000 pg rAPN (P < 0.50) treated cells compared to control [Figure 1b].

**Adiponectin influence vascular endothelial growth factor and pigment epithelial derived factor expression**

Retinal pigment epithelial exposed to rAPN showed decreased VEGF mRNA and increased PEDF mRNA compared with cells not exposed to rAPN [Figure 2a] by reverse transcriptase PCR, which was further confirmed and quantified by RT PCR. GAPDH was run as an internal control.

rAPN treated cells showed 38% decrease (P < 0.01) in VEGF expression in the cells treated with 5.0 pg rAPN, 42% decrease (P < 0.01) in cells treated with 50 pg rAPN, 47% decrease (P < 0.01) in cells treated with 500 pg rAPN, 22% decrease (P < 0.01) in cells treated with 5000 pg rAPN compared to control cells. Whereas, PEDF expression showed 27% increase (P < 0.01) in cells treated with 500 pg rAPN and 41% increase (P < 0.03) in 5.0 ng rAPN treated cells compared with control [Figure 2b].

**Figure 1:** Quantification of vascular endothelial growth factor (VEGF) and pigment epithelial derived factor (PEDF) protein after exposing with various concentration of rAPN. (a) Quantification of VEGF in the conditioned medium showed a significant decrease in rAPN exposed retinal pigment epithelial cells when compared to control (*statistically significant compared to control). (b) Quantification of PEDF in the conditioned medium in rAPN exposed retinal pigment epithelial cells
Intra vitreal and intraperitoneal injection of recombinant APN decreased choroidal neovascularization by 68% and 78% respectively. Our earlier study concur the increased APN exposure showed a mild increase in PEDF protein expression which did not attain statistical significance. However, experiments with longer duration of exposure may help for definite conclusion.

It has been reported that APN is expressed in choroidal blood vessels and it is increased with LASER treatment. Intra vitreal and intraperitoneal injection of recombinant APN decreased choroidal neovascularization by 68% and 78% respectively. Our earlier study concur the increased APN in vitreous of the patients with PDR who underwent LASER treatment prior to surgery. This increase in APN correlates negatively with VEGF and our cell culture experiments resulted in decreased VEGF when exposed to APN as reported by Bora et al. APN suppresses VEGF-stimulated human coronary artery endothelial cell migration via cAMP/PKA-dependent signaling, which shows implications for APN as a regulator of vascular processes associated with diabetes and atherosclerosis.

Adiponectin has been considered as a regulator of vascular remodeling. It is antiangiogenic by (a) decreasing interleukin 8 expression and secretion from EC, (b) forming complex with growth factor like platelet derived growth factor, fibroblast growth factors, and epidermal growth factor (EGF) reducing their bioavailability, (c) inhibiting EC migration and proliferation in vitro and in vivo assays, (d) attenuating hypoxia-induced pathological retinal neovascularization in mice. On the other hand, it also exerts proangiogenic action by (a) activating AMPK and inhibiting caspase 3 in animal and cell culture experiments and (b) promoting blood flow to ischemic limb. APN has been reported to have a pleotropic effect on the maintaining the vascular integrity. At this point we do not know whether APN is pro or anti angiogenic molecule in eye, however, our findings indicate it could inhibit VEGF expression.

Thus, decrease in VEGF protein expression as well as VEGF mRNA expression in rAPN treated cells indicates that APN can modulate the expression of VEGF in cultured ARPE-19 cells.

### Discussion

In this study, we report that APN as an antiangiogenic molecule which modulates the two major angiogenic regulators in cultured ARPE cells. Our in vitro studies on RPE cells exposed to various concentration of recombinant APN showed that APN down regulates VEGF expression and up regulates PEDF gene expression. Recombinant APN exposure showed a mild increase in PEDF protein expression which did not attain statistical significance. However, experiments with longer duration of exposure may help for definite conclusion.

### Conclusion

The present study revealed that APN decreased both VEGF mRNA as well as protein expression in cultured RPE cells. Effective decrease in VEGF indicates that APN plays a protective role against VEGF induced angiogenesis. However, further studies on APN are needed to elucidate therapeutic application.

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