Effects of triamcinolone on the expression of VEGF and PEDF in human retinal pigment epithelial and human umbilical vein endothelial cells

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Purpose: To investigate whether triamcinolone acetonide (TA) affects the expression of vascular endothelial growth factor (VEGF) and pigment epithelial derived factor (PEDF) in human retinal pigment epithelium (ARPE19) and human umbilical vein endothelial (HUVE) cells. Study the time course of the effects of TA on VEGF and PEDF expressions in cultured cells.

Methods: ARPE19 and HUVE cells were grown to subconfluence and treated with TA (0.1 mg/ml, 1 mg/ml). The mRNA expressions of VEGF and PEDF were determined from 10 min to three days using real-time RT-PCR. Concurrently, the protein levels of VEGF and PEDF in ARPE cells were detected with ELISA.

Results: Real-time RT-PCR showed TA affected a 0.5 fold decrease in VEGF level and about a 2.5 fold increase in PEDF level at both TA concentrations. The effect was maintained at 12 h at 0.1 mg/ml TA and 24 h at 1 mg/ml TA. Similar changes were observed in the respective protein concentrations. The effects of TA on VEGF and PEDF transcript levels were similar in HUVE and ARPE19 cells. VEGF and PEDF protein productions in HUVE cells were too low for statistical analysis.

Conclusions: TA reduces the expression of VEGF but increases the expression of PEDF in ARPE19 and HUVE cells. These observations suggest TA may influence the inhibition of neovascularization and macular edema through differential VEGF and PEDF expressions.
served to regulate PEDF expression in mouse Muller glial cells and rat glioma cells [22]. A recent study shows that human umbilical venous endothelial (HUVE) cells expressed angiogenesis factors including VEGF and PEDF [23].

We have previously shown that TA caused stress responses of cultured ARPE19 cells [24]. In this study we hypothesized that TA may affect the expressions of VEGF and PEDF in retinal pigment epithelium (RPE) cells and vascular endothelial cells to stabilize CNV and reduce macular edema. We therefore sought to investigate whether TA affects VEGF and PEDF in cultured human retinal pigment epithelium (ARPE19) and HUVE cells.

METHODS

Cell culture: ARPE19 and HUVE cell lines were purchased from the American Type Culture Collection (Manassas, VA). Cell culture reagents, fetal bovine serum, and chemicals came from Invitrogen-Gibco (Rockville, MD), and containers from Corning Glass (Acton, MA).

Human ARPE19 cells were grown in 1:1 (vol/vol) mixture of Dulbecco’s modified Eagle’s and Ham’s F12 medium (DF), containing 3 mM L-glutamine, 10% fetal bovine serum, and antibiotic mixtures of 100 U/ml penicillin G and 100 µg/ml streptomycin sulfate (Invitrogen-Gibco, Rockville, MD). HUVE cells were grown in F12K medium, containing 0.1 mg/ml heparin, 20% fetal bovine serum, 0.03 mg/ml endothelial cell growth supplement (BD Biosciences, San Jose, CA), and antibiotic mixtures of 100 U/ml penicillin G and 100 µg/ml streptomycin sulfate. Both types of cells were seeded onto 100 mmx20 mm plates. The cultures were maintained in a humidified 5% CO₂ environment at 37 °C. All the cells within the same passages were grown to 70% confluence for TA treatment.

Treatment with triamcinolone: Cells were grown to 70% confluence, and cell cultures were adapted into fresh culture medium 12 h prior to addition of triamcinolone acetonide (TA, 9α-fluoro-16α-hydroxyprednisolone; kenacort-A, Bristol-Myers-Squibb, NY), which was serially diluted in culture medium to appropriate concentrations just before use. TA (0.1-1 mg/ml) and vehicle (benzyl alcohol, 0.025%) were added to the ARPE19 and HUVE cells. Treated and nontreated cells and their culture medium were collected at 0, 10 min, 30 min, 1 h, 3 h, 6 h, 12 h, 24 h, 2 d, and 3 d for RNA extraction, real-time RT-PCR and ELISA analysis. All experiments were performed at least twice and all time point experiments collected in triplicate. The culture medium used in these experiments was all freshly prepared and contained all ingredients as well as containing the cells.

The 3-(4,5-dimethyiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to examine the effects of TA on cell proliferation as previously described [24].

RNA isolation and real-time RT-PCR: Total RNA was extracted with a RNeasy kit (Qiagen GmbH, Hilden, Germany). Cells were lysed in lysis buffer containing 1% α-mercaptoethanol (Sigma, St.Louis, MO) and passed through a separation column (QiASHredder; Qiagen GmbH, Germany). Total RNA was obtained according to the supplier’s protocol and quantified with a spectrophotometer (NanoDrop). For reverse-transcription 500 ng total RNA was used with 3 µg/µl random primer (dN)6 (Roche Diagnostics GmbH, Mannheim, Germany) and a reverse transcriptase kit with RNase inhibitor (Superscript™ Reverse Transcriptase Kit and RNase OUT RNase inhibitor) purchased from Invitrogen (Carlsbad, CA).

The amount of cDNA corresponding to 25.0 ng RNA was selected and amplified with the following primer pairs: GAPDH forward, 5'-gaa gtt gag gtt cgg agt-3', and reverse, 5'-gaa gat ggt gag ggt att tc-3'; VEGF<sub>165</sub> forward, 5'-gac aag aaa atc ctt gtg gcc-3', and reverse, 5'-acc agc aag ctt tgt ttt tgc-3'; PEDF forward: 5'-cag aac aec aag ctc ggc-3', and reverse, 5'-ctt cat cca aag aat aat cct c-3'. We also measured the transcript expression of VEGF isoforms: VEGF<sub>165</sub>, VEGF<sub>121</sub>, VEGF<sub>189</sub>, with primer pair: forward 5'-atc ttc aag cca ttc tgt ggc cc-3', and reverse 5'-tca ccg cct cgg ctt gtc aca t-3'. Real-time RT-PCR analysis was performed using SYBR Green PCR master mix (Applied Biosystems, Foster City, CA). The relative quantification was normalized to the GAPDH gene expression level.

The ABI PRISM® 7000 Sequence Detection System was used for real-time detection of PCR and data analysis. The mean Ct value (threshold cycle; cycle at which the increase in signal associated with exponential growth of PCR product was first detected) of the stimulated sample was compared to that of the unstimulated control sample, using the Ct value of GAPDH as an internal control. ΔCt was the difference in Ct values derived from the target gene (in each sample assayed) and the GAPDH gene, while ΔΔCt represented the difference between the paired samples. The n-fold differential ratio was expressed as 2<sup>ΔΔCt</sup>.

Enzyme-linked immunosorbent assay (ELISA) for VEGF<sub>165</sub> and PEDF: Concentrations of VEGF<sub>165</sub> and PEDF in the medium of the cultured ARPE19 cells were determined by an enzyme-linked immunosorbent assay (ELISA, ChemiKine, Chemicon International). Duplicate wells were used for all samples and standards.

Statistical and data analysis: The effects of TA on the expression of VEGF and PEDF between the control (vehicle) and TA-treated groups were analyzed with ANOVA. Significant differences were determined between control and TA-treated cells at the respective time points of sample collections. In all the experiments, p<0.05 was considered to indicate a statistically significant difference.

RESULTS

Effect of TA on VEGF<sub>165</sub> and PEDF mRNA expression in RPE cells: TA was added to the culture medium of the ARPE19 cells at two concentrations (0.1 mg/ml and 1 mg/ml). Real-time RT-PCR measurements showed significant alterations in VEGF<sub>165</sub> and PEDF mRNA expressions in triamcinolone-treated ARPE19 cells (Figure 1). The maximum alterations occurred at approximately 1-3 h in all cases, a 0.5±0.05 fold decrease in levels of VEGF<sub>165</sub> (p=0.00012) and 2.5±0.07 fold increase (p<0.0001) in levels of PEDF. The amplifications showed no significant difference between the two concentrations of TA, but the effect was maintained till 12 h (p=0.00018).
Effect of triamcinolone acetonide on VEGF<sub>165</sub> and PEDF mRNA expression in RPE cells: To examine whether the increased expression of VEGF<sub>165</sub> and PEDF mRNA was accompanied by changes in protein production, expression of VEGF<sub>165</sub> and PEDF protein production in RPE cells was measured.

Figure 1. Effects of TA on VEGF<sub>165</sub> and PEDF mRNA expression in RPE cells. Effect of triamcinolone acetonide (TA) on vascular endothelial growth factor (VEGF<sub>165</sub>, panel A) and pigmented epithelium derived factor (PEDF, panel B) mRNA expression in RPE cells. TA (0.1 and 1.0 mg/ml) was added to the culture medium. Real-time RT-PCR measurements (mean±SD) indicated a 0.5 fold decrease in levels of VEGF<sub>165</sub> and a 2.5 fold increase in levels of PEDF. The amplifications showed no significant difference between the two concentration of TA, but the effect was maintained till 12 h by 0.1 mg/ml TA and till 24 h by 1.0 mg/ml TA. *p<0.005, control drug-free growth versus TA-treated cultures by ANOVA.

Figure 2. Effects of TA on VEGF<sub>165</sub> and PEDF protein production in RPE cells. Effect of triamcinolone acetonide (TA) on vascular endothelial growth factor (VEGF<sub>165</sub>, panel A) and pigmented epithelium derived factor (PEDF, panel B) protein production in RPE cells. ELISA was performed on culture medium of ARPE19 cells. Assay of VEGF<sub>165</sub> and PEDF protein (mean±SD) for different time periods indicated that the protein levels of VEGF<sub>165</sub> and PEDF were increased or decreased starting at 3 h after TA addition. *p<0.005, control drug-free growth versus TA-treated cultures by ANOVA.

After 0.1 mg/ml TA stimulation and till 24 h (p=0.0007) after 1 mg/ml TA stimulation (Figure 1). Expression level of VEGF<sub>121</sub> mRNA was similar to VEGF<sub>165</sub> but only weak VEGF<sub>189</sub> mRNA expression was obtained.
VEGF<sub>165</sub> and PEDF in the culture medium were assayed by ELISA. There was a significant decrease in protein levels of VEGF<sub>165</sub> (p=0.001) and an increase in PEDF (p=0.002) starting at 3 h after TA addition (Figure 2).

**Expression studies of VEGF<sub>165</sub> and PEDF in HUVE cells:**

The effects of TA on VEGF<sub>165</sub> and PEDF transcript levels in HUVE cells were similar to those in ARPE19 cells. Quantitative RT-PCR measurements indicated a maximum reduction of 0.5 fold±0.05 in VEGF<sub>165</sub> mRNA levels (p=0.0006) and a 2.2 fold±0.06 increase in PEDF mRNA levels (p=0.00005) 3 h after TA treatment in HUVE cells. The two different concentrations of TA (0.1 mg/ml and 1 mg/ml) had a similar effect (Figure 3). However, in contrast to real-time RT-PCR results, no difference in VEGF and PEDF protein expressions were observed in HUVE cells after TA treatments. The protein levels were too low for statistical analysis, less than 100 pg/ml for VEGF<sub>165</sub> and undetectable (<1 ng/ml) for PEDF (data not shown).

**DISCUSSION**

Recently, intravitreal injection of TA has received much attention as a therapeutic modality for CNV and macular edema. Many clinical studies indicated that this approach is safe and effective [3-11]. Matsuda et al. showed that TA reduced VEGF expression and induced CTGF expression in ARPE19 cells exposed to oxidative stress [25]. In the present study, VEGF mRNA expression was examined in normal cultured human ARPE19 cells. We also measured the transcript expression of the VEGF isoforms VEGF<sub>165</sub>, VEGF<sub>121</sub>, and VEGF<sub>189</sub> in our preliminary experiments. RPE cells expressed strong VEGF<sub>165</sub> and 121, and the expression patterns and levels were similar. Only VEGF<sub>189</sub> was weakly expressed. VEGF<sub>165</sub> is the major VEGF isoform and is the most abundant and biologically active [26]. VEGF<sub>121</sub> is biologically active in endothelial cells, but has lower potency than VEGF<sub>165</sub>. Therefore, we only measured VEGF<sub>165</sub> transcript levels in our TA experiments. Our real-time quantitative analysis revealed that TA decreased the expression of VEGF in RPE cells at both the mRNA and protein levels (Figure 1 and Figure 2). This is consistent with the finding that glucocorticosteroids can reduce VEGF mRNA and protein expression in cultured eosinophils [27] as well as reduce VEGF in cultured aortic vascular smooth muscle cells [28]. Countereffects of corticosteroids on VEGF have also been demonstrated in a recent rabbit study, in which intravitreal VEGF injections caused a time and dose-dependent breakdown of blood-retina and blood-aqueous barriers and led to vascular leakage. The breakdowns were blocked both by dexamethasone and TA, but not the nonsteroidal anti-inflammatory drug (NSAID) indomethacin [29].

It is possible that TA inhibits the angiogenesis effects of VEGF downstream from VEGF receptors or TA enhanced the expression of PEDF, which is a potent inhibitor of angiogenesis. VEGF has the ability to increase vascular permeability, which causes leakage of proteins and other molecules out of blood vessels [30]. Enhanced PEDF production may disrupt VEGF-induced vascular permeability. VEGF-driven angiogenesis likely plays a major role in the pathogenesis of CNV [14]. Clinical trials of anti-VEGF therapy, such as anti-VEGF aptamer and anti-VEGF antibody fragment, have also shown their efficacy on CNV [31,32]. VEGF was originally identified as vascular permeability factor (VPF) as a result of its potent ability to increase vascular permeability, resulting in leakage of proteins and other molecules from blood vessels [15,30]. Such permeability change in blood vessels may be a cause of macular edema. This leakage effect by VEGF has been demonstrated in the breakdown of blood-retinal barrier.
in a rabbit model, which could be inhibited by corticosteroids [29]. Although the exact effect of steroids on VEGF expression in RPE cells in vivo is still unclear, our results suggest that TA may reduce CNV and macular edema through regulating VEGF expression in RPE cells. Expressions of VEGF and PEDF have been detected in HUVE cells, and PEDF may help maintain the expression of VEGF-C under hypoxic conditions. [23] We also found that the effects of TA on HUVE cells were similar to those in ARPE19 cells (Figure 3), indicating the regulation of VEGF and PEDF expressions by TA on vascular endothelial cells.

Contrary to VEGF, TA increased the PEDF expression in cultured human ARPE19 cells (Figure 1 and Figure 2). A recent study reported dexamethasone can regulate the expression of PEDF in murine Muller glial cells and C6 rat glioma cells [22]. Uchida et al. found that vitamin A upregulated the expression of PEDF in RPE cells [20]. PEDF is one of the most potent inhibitors of angiogenesis so far described [33,34]. It also promotes cell differentiation in normal retinal cells, maintains morphological organization of the differentiated retina, and promotes photoreceptor outer segment formation and maturation [35-37]. It has been shown that VEGF secreted by RPE cells upregulated PEDF expression in an autocrine manner [38]. In present study, TA downregulated the expression of VEGF and upregulated the expression of PEDF at the same time. Therefore, TA may prevent angiogenesis by both suppressing VEGF production and stimulating PEDF expression. This may also be one explanation for the beneficial clinical effects of TA, since high levels of PEDF can protect retinal photoreceptors and neurons and thus may result in favorable clinical outcome.

In conclusion, we demonstrated that TA reduces the expression of VEGF, but induces the expression of PEDF in APRE 19 cells. Similar effects were observed for their mRNA levels in HUVE cells. Although the detailed interactions between these molecules remain to be elucidated, our findings suggest VEGF and PEDF is involved in the mechanism by which TA influences the inhibition of CNV and macular edema.

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