Mineralocorticoids Restore Quiescent Morphology and Reduce VEGF Receptor Expression in Inflamed Choroidal Endothelial Cells in vitro

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Abstract
Background/Aims: While the glucocorticoid triamcinolone acetonide (9α-fluoro-16α-hydroxyproglandinone, TA) has been widely administered as a treatment of ocular inflammation, mineralocorticoids have not been tested for their efficacy. Methods: We assessed cellular morphology and actin distribution by immunofluorescence and light microscopy, membrane permeability with transendothelial resistance and cell surface vascular endothelial growth factor receptor-1 (VEGFR-1) expression by flow cytometry. Results: Fludrocortisone acetate was more effective than TA in restoring quiescent morphology and reducing membrane permeability in phorbol-12-myristate-acetate (PMA)-stimulated choroidal endothelial cells (CECs). Each of the corticosteroids inhibited VEGF-R1 cell surface expression in PMA-responsive CECs. Conclusion: Mineralocorticoids may be of potential use in reducing vascular permeability in ocular disease.

Introduction
Inflammation of ocular tissues is a key component of many diseases of the eye and is characterised by breakdown of the blood-retinal barrier due to increased permeability of the endothelial lining. The resulting reduced barrier function has been associated with changes in the expression and function of the pro-inflammatory factor, vascular endothelial growth factor (VEGF), and its receptors [1-4]. In normal adult human eyes in vivo, VEGF receptors including VEGF-R1 (flt-1) and VEGF-R2 (flik-1) are located predominantly on the choriocapillaris endothelium, presumably enabling response to VEGF produced by the retinal pigment epithelium [5]. In vitro, VEGF receptors have been detected at the mRNA and protein level in a range of endothelial preparations, although results vary substantially from study to study [4, 6-10]. When cell barrier permeability is increased in vivo or in vitro, VEGF and its receptors are upregulated and contribute to the underlying changes in endothelial cell adhesion, thereby aggravating the inflammatory consequences [2, 3, 11].

One of the most widely administered intravitreal agents for the treatment of ocular inflammatory diseases is the glucocorticoid triamcinolone acetonide (9α-fluoro-
16α-hydroxyprogrenolone, TA) [12]. Binding of corticosteroids to their receptors results in active Na+ re-absorption, and improved cell barrier function [13], which leads to favourable outcomes on inflammation and ocular edema [12, 14]. However, there are limited numbers of studies of the effects of corticosteroids on markers of vascular permeability such as VEGF and its receptors. The only study to date suggests that up-regulation of VEGF expression in Muller cells stimulated with IL-1β can be prevented at the transcriptional level by application of the glucocorticoid TA [15]. VEGF receptor levels were not assessed in the study. Moreover, concerns have been raised regarding the cytotoxicity of TA for photoreceptors and retinal pigment epithelial cells [16, 17].

By contrast, other corticosteroids such as the mineralocorticoids have not been tested for their efficacy in treating the symptoms of ocular inflammation, despite the high levels of expression of the mineralocorticoid receptor in ocular tissues making them promising candidates [18]. Mineralocorticoids are characterised by homology to aldosterone, the primary endogenous mineralocorticoid, and by their ability to alter mineral balance [13]. The present study was designed to compare TA with a range of mineralocorticoids in their ability to modulate the actions in vitro of the immunogenic stimulant phorbol-12-myristate acetate (PMA) on choroidal endothelial cells (CECs). Stimulation of endothelial cells in vitro with PMA mimics the altered vascular permeability observed in ocular inflammation, producing substantial barrier dysfunction, accompanied by redistribution of actin and elevation of VEGF and VEGF receptor expression [19]. We show that fludrocortisone acetate (FCA) is more potent than TA in restoring CECs to a quiescent morphology following PMA stimulation. Membrane permeability as measured by trans-endothelial resistance (TER) reflected morphological changes. Furthermore, each of the corticoids tested significantly reduced VEGF-R1 expression in stimulated CECs.

Methods

Cell Culture

CECs were seeded on day 0 into transwell units of 0.4 μm pore size, 12 mm diameter (Millipore) at a density of 6.67 × 10^4 cells/cm². Cells were cultured in DMEM/F12 supplemented with 10% FCS for 3 days, the media changed and replaced with fresh DMEM/F12 supplemented with 10% FCS. On day 6, the media were replaced with DMEM/F12 supplemented with 1% FCS. At this time, CECs were stimulated with 1 μM PMA and treated with DOCA (10 μM), FCA (10 μM) or TA (50 μM). On day 7, TER was measured using an EndOhm-6 voltmeter (World Precision Instruments). Background resistance of the membrane alone was subtracted from all values, and results were expressed as unit area resistance (ohm-square centimetre).

Flow Cytometry

48 h after stimulation, cells were rinsed with PBS (Ca²⁺ - and Mg²⁺-free PBS) supplemented with 0.5% bovine serum albumin (Sigma, PBS + 0.5% BSA) and detached from the wells using Accutase (Chemicon) for 15 min at 37°C. The cells were washed in PBS + 0.5% BSA and stained with anti-human VEGF-R1 (flt-1)-allophycocyanin (APC, R&D Systems) at 3.75 μg/ml for 60 min at 4°C in the dark. Cells were washed 3 times in PBS + 0.5% BSA before flow-cytometric analysis. Populations of 10,000 live cells were gated on forward versus side scatter plots and analysed for APC fluorescence. Background fluorescence of unstained cells was subtracted and the results expressed as a percentage of VEGF-R1-positive cells.

Study of the literature revealed a dependency of VEGF receptor expression on serum concentrations in culture [8, 20]. Preliminary flow cytometry experiments showed that the strongest
increase in VEGF-R1 expression on the surface of CECs, in response to PMA (1 μM), occurred in the presence of 1% FCS (increased from 8.2 to 41.4%). CECs treated with PMA (1 μM) in the presence of 10% FCS had significantly reduced viability and reduced expression of VEGF-R1 (reduced from 62.2 to 26.4%). While CECs treated with 1 μM PMA in the absence of serum had increased VEGF-R1 expression (increased from 33.6 to 68.2%), cell viability was significantly compromised. Therefore, further experiments on the effects of mineralcorticoids on VEGF-R1 expression were carried out in DMEM/F12 + 1% FCS. No significant VEGF-R2 expression was detected in the presence of any of the serum or PMA concentrations tested (0.1, 1 or 10 μM, results not shown). Similar experiments were carried out on retinal pigment epithelial cells (RPE-1, ATCC: CRL-2240), but consistent levels of cell surface expression of VEGF-R1 or VEGF-R2 were not demonstrated (results not shown).

Data Analysis

Data are expressed as mean ± SEM and were analysed by single-factor ANOVA followed by Tukey, Fischer or Bonferroni/Dunn post hoc tests (as detailed in Results) to determine statistical significance between pairs of means.

Results

Effects of Corticosteroids on CEC Morphology

Untreated CECs exhibited cobblestone morphology with a slightly raised and regular confluent appearance (fig. 1A). By contrast, CECs stimulated with PMA were flatter with long thin processes and a proportion of the cells were rounded and granular, reflecting the expected increased detachment and reduced viability (fig. 1B). FCA (fig. 1C) and to a lesser extent TA (fig. 1D) application abrogated the effects of PMA treatment, with cells displaying a smoother and confluent morphology typical of quiescent endothelial cells. Nonetheless, characteristic cobblestone morphology of untreated CECs was not entirely restored (fig. 1A, C, D). Toxic effects were not observed in our study, as previously reported in response to aggregations of TA in the commercially available preparations [12]. PMA-stimulated CECs treated with 11-DOC, DOC and DOCA had similar morphology to untreated PMA stimulated CECs (fig. 1E–G).

We conducted a time course study to ascertain if the effects of TA and FCA on CEC morphology could be heightened with pre-treatment (24 h before PMA stimulation), or could still be detected if treatment was delayed for 24 h after PMA stimulation. Pre-treatment with FCA (fig. 2A) was more effective in preventing the effects of PMA compared to coincident (fig. 2C) or delayed treatment (fig. 2E). Similarly, pre-treatment of CECs with TA increased the positive effects on cell morphology, although not resulting in return of normal cobblestone morphology (fig. 2B), whereas coincident (fig. 2D) and delayed (fig. 2F) treatment were not as effective. FCA treatment of CECs generally resulted in more confluent and morphologically quiescent cells than following treatment with TA at each of the treatment times (fig. 2).

Effects of Corticosteroids on F-Actin Distribution

We wanted to determine if the effects of TA and FCA on PMA-stimulated CEC morphology were accompanied by changes in F-actin distribution. Stimulation of CECs with PMA resulted in a striking rearrangement of F-actin from uniform strands throughout the cells to a punctate distribution predominantly at the cell periphery, indicating disruption of the cytoskeleton. The rearrangement took place within 30 min of PMA stimulation (fig. 3A, B). Treatment of PMA-stimulated CECs with either FCA (fig. 3C) or TA (fig. 3D) reduced the number of cells with peripherally aggregated F-actin and resulted in flattened less spindly-shaped CECs.

Effects of Corticosteroids on TER

In order to assess the functional effects of corticosteroids on PMA-stimulated CECs, the TER of PMA stimulated CECs was assessed following treatment. As expected, stimulation of CECs with PMA reduced the resistance of untreated CECs (p ≤ 0.0001, fig. 4). Treatment of PMA stimulated CECs with FCA raised the resistance of these cultures (p ≤ 0.05). We were unable to detect any effects of TA or DOCA on resistance of PMA-stimulated CECs (fig. 4).

Corticosteroids Reduced VEGF-R1 (Flt-1) Protein in CECs

Our findings on the effects of corticosteroids on CEC morphology and function led us to speculate on the potential mechanism by which corticosteroids were reducing the inflammatory morphology and membrane permeability induced by PMA stimulation. We hypothesised that corticosteroids may be acting via the VEGF receptor system. Accordingly, we assessed the effects of these corticosteroids on VEGF receptor protein expression at the cell surface.

The corticosteroids TA, 11-DOC, DOC, DOCA or FCA were tested for their efficacy at reducing expression of VEGF-R1 protein in response to PMA stimulation of CECs (fig. 5). ANOVA demonstrated a significant difference between treatment groups (p ≤ 0.0001). Multiple comparison tests demonstrated significant abrogation of the stimulatory effects of PMA on VEGF-R1 expression.
in response to treatment with 50 μM TA, 50 μM DOC, 10 μM and 50 μM DOCA and 10 μM FCA (p ≤ 0.05). A slight biphasic effect of FCA was shown, with 50 μM not significantly inhibiting PMA-stimulated VEGF-R1 expression.

A time course of addition was performed in order to determine whether addition of corticosteroids prior to or after PMA stimulation resulted in similar reductions in VEGF-R1 expression (fig. 6). CECs treated with each of the corticosteroids expressed reduced levels of VEGF-R1 compared to untreated PMA-stimulated cells, regardless of the time of addition of the corticosteroid (p < 0.05). There were no differences in VEGF-R1 expression between treatment times for each corticosteroid (p > 0.05).
Fig. 2. Effects on cell morphology of treating CECs with an optimal dose of corticosteroids before or after stimulation. A CECs without PMA stimulation. B CECs with 1 μM PMA stimulation. CECs were stimulated with 1 μM PMA 24 h after plating (B-H) and treated with corticosteroids either at the time of plating, with the PMA or 24 h after PMA stimulation. Cells were photographed 72 h after plating and representative images are shown. C, E, G CECs treated with 10 μM FCA at time 0, 24 and 48 h, respectively. D, F, H CECs treated with 50 μM TA at time 0, 24 and 48 h, respectively. 10x magnification, scale bar = 50 μm.

Reductions in VEGF-R1 were seen in response to 50 μM 11-DOC (fig. 6, p < 0.05).

Avastin Enhanced the Effects of Corticosteroids on VEGF-R1 Expression

In order to determine if avastin could potentiate the effects of corticosteroids on cell surface VEGF-R1 expression, the effects of these agents on VEGF-R1 expression were assessed with or without avastin. Addition of avastin caused greater reductions in VEGF-R1 than those produced by DOCA (p = 0.0016), FCA (p = 0.0398) or TA (p ≤ 0.0001) alone (fig. 7A). Treatment of PMA-stimulated CECs with avastin alone also reduced VEGF-R1 expression (9.3% reduction, p = 0.0198). The effects of avas-
tin plus DOCA (24.2% reduction) and of avastin plus FCA (25.5% reduction) were approximately equal to the additive effects of these individual treatments. However, the magnitude of the reduction in VEGF-R1 in response to TA plus avastin (39.9% reduction) was greater than the effect of either agent alone (fig. 7A).

**Spirolactone Abrogated the Effects of TA on VEGF-R1 Expression**

The mineralocorticoid receptor inhibitor spirolactone was used in order to determine if the effects of corticosteroids on cell surface VEGF-R1 expression by PMA-stimulated CECs were mediated by the corticosteroid receptor. Treatment of PMA-stimulated CECs with spirolactone (10 μM) alone did not significantly affect VEGF-R1 expression (fig. 7B). However, the reduction in VEGF-R1 expression in response to TA treatment of PMA-stimulated CECs was abrogated by spirolactone. VEGF-R1 expression by PMA-stimulated CECs treated with TA plus spirolactone was not significantly different from PMA-stimulated untreated cells (p = 0.7544) (fig. 7B). Similarly, the reduction in VEGF-R1 expression in response to FCA was partially abrogated by spirolactone. VEGF-R1 expression by PMA-stimulated CECs treated with FCA plus spirolactone was not significantly different from PMA-stimulated untreated cells (p = 0.4688) (fig. 7B).

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Mineralocorticoids Restore Endothelial Quiescence

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Fig. 4. Membrane permeability (TER) of PMA-stimulated CECs treated with corticosteroids. All treatment groups except 'unstimulated' were stimulated on day 6 after plating with 1 μM PMA and treated with DOCA (10 μM), FCA (10 μM) or TA (50 μM) as designated on the x-axis. TER was performed on day 9 after plating: background resistance of the membrane alone was subtracted from all values and results were expressed as the mean ± SEM unit area resistance. Statistical analyses by single-factor ANOVA followed by Fisher's multiple comparison tests were performed to determine statistical significance (* p < 0.05) between untreated and treated groups for each corticosteroid.
Fig. 5. Effects of varying doses of mineralocorticoids on VEGF-R1 expression by CECs. Cells were stimulated with 1 μM PMA and concurrently treated with corticosteroids at 24 h after plating. Cell surface expression of VEGF-R1 was assessed by flow cytometry 72 h after plating. Results are expressed as mean ± SEM percentages of VEGF-R1-positive cells and were analysed by single-factor ANOVA followed by Tukey multiple comparison tests to determine statistical significance (* p < 0.05) between untreated and treated groups for each corticosteroid.

Fig. 6. Effects of varying the time of corticosteroid treatment on VEGF-R1 expression by CECs stimulated with PMA. Cells were stimulated with 1 μM PMA 24 h after plating and treated with TA, 11-DOC or DOC at 50 μM or DOCA or FCA at 10 μM, either at the time of plating (T0), with PMA stimulation (T24) or 24 h after PMA stimulation (T48). Cell surface expression of VEGF-R1 was assessed by flow cytometry 72 h after plating. Results are expressed as mean ± SEM percentages of VEGF-R1-positive cells and were analysed by single-factor ANOVA followed by Tukey multiple comparison tests to determine statistical significance (* p < 0.05) compared to untreated CECs for each corticosteroid.

Fig. 7. Effects of avastin or spironolactone on cell surface VEGF-R1 expression by corticosteroid-treated CECs. A All treatment groups except 'unstimulated' were stimulated 24 h after plating with 1 μM PMA and treated with DOCA (10 μM), FCA (10 μM) or TA (50 μM) with or without 0.8 mg/ml avastin as designated on the x-axis. Flow-cytometric analysis of cell surface VEGF-R1 expression was performed 72 h after plating. Results are expressed as means ± SEM percentages of VEGF-R1-positive cells. Statistical significances between groups treated with corticosteroids or with corticosteroids plus avastin were determined using ANOVA and Bonferroni/Dunn post hoc tests (* p < 0.05). B VEGF-R1 expression was analysed as described in A, but in response to corticosteroids with or without 10 μM spironolactone. Statistical significances between PMA-stimulated but untreated groups and groups that were PMA stimulated and treated with corticosteroids or with corticosteroids plus spironolactone were determined using ANOVA and Bonferroni/Dunn post hoc tests (* p < 0.05; NS is not significantly different from PMA-stimulated untreated CECs).
Discussion

In summary, we have demonstrated that selected mineralocorticoids are as good as, or in the case of FCA more effective than, the glucocorticoid TA in preventing the cellular changes associated with increased permeability of the blood-retinal barrier.

Selected Corticosteroids Prevent Inflammatory Changes in Cell Morphology and Cytoskeleton via a Receptor-Independent Pathway

Alterations in endothelial cell morphology resulting in disruption of endothelial cell monolayers in culture are indicative of alterations in membrane permeability [21]. The mineralocorticoid FCA, particularly when administered prior to immune stimulation, restored CECs to a typical cobblestone monolayer morphology following PMA stimulation. Endothelial cells are tethered to each other and the underlying substrate through adhesion molecules linked to the F-actin cytoskeleton [22]. The focal adhesion contacts are crucial in maintaining the blood-retinal barrier and preventing leukocyte migration and subsequent inflammation [23]. Alterations in the cytoskeletal arrangements of F-actin in response to PMA were at least partially abrogated by FCA and TA. These effects were reflected in measures of TER that demonstrated the capacity of FCA to improve CEC barrier function. The results suggest that FCA would be more effective than TA at restoring barrier function to CECs and thereby reducing ocular inflammation.

The activity of mineralocorticoids is induced via mineralocorticoid receptors, nuclear hormone receptors that regulate gene expression, as well as via receptor-independent pathways that remain poorly characterised. Mineralocorticoid receptors are widely distributed in tissues of diverse embryological origins [13]. They are located cytoplasmically in association with the cytoskeleton, particularly tubulin [24]; the interaction is thought to be critical for receptor function. Disruption of the cytoskeleton (either F-actin or tubulin) can eliminate receptor-mediated activity of mineralocorticoids [18]. The result raises the possibility that the effects of mineralocorticoids on cell morphology in our model are receptor independent since PMA stimulation rapidly disrupted F-actin organisation in CECs. Furthermore, the changes induced by mineralocorticoids occurred too rapidly (30 min) to be caused by changes in gene expression. In addition, spironolactone had no impact on TER values following PMA treatment (data not shown). Mineralocorticoid receptor-independent action of mineralocorticoids is thought to occur via direct interactions of the drugs with the cytoskeleton [24].

Mineralocorticoids Reduce VEGF-R Expression via a Receptor-Dependent Pathway

Our results suggest that mineralocorticoids also act via the mineralocorticoid receptor to reduce VEGF-R1 expression in CECs stimulated with PMA. However, reductions in VEGF-R1 expression did not consistently correlate with improvements in morphology. These findings suggest separate mechanisms for modulation of PMA-induced changes in cell morphology (mineralocorticoid receptor independent) and inflammatory marker expression (mineralocorticoid receptor dependent). For example, treatment of PMA-stimulated CECs with DOCA resulted in decreases in the inflammatory marker VEGF-R1 expression, but did not change the PMA-induced rounding and retraction of these cells. Analysis of the distribution of tight junction proteins may provide additional information about the functional state of CECs in response to corticosteroid treatment and give clues as to the possible pathways by which these agents produce morphological changes.

The total abrogation of the effects of the glucocorticoid TA on VEGF-R1 expression following administration of the mineralocorticoid receptor antagonist spironolactone was initially surprising. However, spironolactone has been shown to display some antagonistic activity towards the glucocorticoid receptor [25]. The lack of a significant difference between FCA-plus-spironolactone-treated PMA-stimulated cells and PMA-stimulated untreated cells support a mineralocorticoid receptor-dependent mechanism for FCA in these effects on VEGF-R1 expression.

It is interesting that bevacizumab (avastin), an antibody raised against VEGF, significantly enhanced the corticosteroid-induced decreases in VEGF-R1 expression. It is likely that avastin bound any endogenous VEGF produced by the CEC cells, leading to decreased VEGF receptor expression [6]. The effects of TA plus avastin on VEGF-R1 expression appeared to be synergistic, while the effects of DOCA plus avastin and of FCA plus avastin were approximately additive. Such combinations may affect both the steroid-dependent and -independent pathways involved in inflammation and oedema and may indicate a potential steroid-sparing regimen, allowing lower doses of corticosteroid to be used.
Conclusion

Regardless of the mechanism(s) by which FCA and TA exert their effects on CECs, it seems that FCA could be more effective than TA in decreasing membrane permeability, altering the balance of ocular inflammatory disease and potentially providing a favourable clinical outcome.

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