EXTENDED REPORT

Triamcinolone acetonide suppresses early proangiogenic response in retinal pigment epithelial cells after photodynamic therapy in vitro

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Objective: To investigate the expression of proangiogenic and antiangiogenic factors, vascular endothelial growth factor (VEGF) and pigment epithelium-derived factor (PEDF) in retinal pigment epithelial (RPE) cells after photodynamic therapy (PDT), especially focusing on their change in the presence of triamcinolone acetonide.

Methods: Firstly, the cellular uptake of verteporfin was quantified after confluent ARPE-19 (human retinal pigment epithelial) cells were exposed to 5 µg/ml verteporfin combined with or without 1 µg/ml triamcinolone acetonide for 1 h. Secondly, ARPE-19 cells exposed to various doses of verteporfin were irradiated with 120 mJ/cm² light. After incubation with or without 1 µg/ml triamcinolone acetonide for 2 days, cell viability and expressions of VEGF and PEDF were assessed.

Results: Cellular uptake of verteporfin was not significantly changed by the presence of 1 µg/ml triamcinolone acetonide. In addition, 0.01–0.1 µg/ml of verteporfin showed a dose-dependent toxicity on the ARPE-19 cells 2 days after the light exposure. The presence of verteporfin at a concentration of 0.01 µg/ml did not affect the cell viability but significantly increased VEGF ($p < 0.001$) and reduced PEDF ($p = 0.03$) expression. Administration of triamcinolone acetonide significantly suppressed both this increase in VEGF ($p < 0.001$) and decrease in PEDF ($p = 0.001$).

Conclusions: VEGF was increased and PEDF reduced in cultured RPE cells shortly after PDT even at a sublethal dose. Triamcinolone acetonide suppressed this proangiogenic response.

Photodynamic therapy (PDT) with verteporfin is effective for reducing the risk of moderate or severe vision loss in patients with subfoveal choroidal neovascularisation (CNV) due to age-related macular degeneration.^{1–3} However, a single treatment is not sufficient to achieve complete CNV closure in most cases, requiring multiple treatments with a mean number of treatment of 5.6 over 2 years.³ To reduce the number of treatments required and to achieve more efficient CNV closure, several investigators advocate the use of other antiangiogenic drugs in combination with PDT.^{4–5}

Triamcinolone acetonide is an intermediate-acting corticosteroid suspension harbouring antiangiogenic properties. Several pilot studies suggested that when PDT is performed in combination with intravitreal triamcinolone acetonide injection, visual improvement is better and that an earlier cessation of fluorescein leakage from the lesion is observed compared with PDT alone.^{4–7} The combination treatment also requires fewer treatments to achieve success compared with PDT monotherapy.⁴

Some previous investigations showed that PDT use does not generally cause a marked response of retinal pigment epithelial (RPE) cells in a clinical setting, as assessed by histopathological or immunohistological studies.^{8–9} However, clinical studies have shown that there is a transient increase in the exudative retinal change immediately after PDT.¹⁰ This is presumed to be due to blood–retinal barrier breakdown induced by functional damage to retinal pigment epithelial cells.¹¹ Some clinical investigations and case presentations have indicated the presence of RPE dysfunction, which leads to temporary exacerbation of exudative change¹¹ or irreversible atrophy^{12–13} shortly after PDT. Histological examination of surgically removed subretinal membranes after treatment with verteporfin showed degeneration, metaplastic change and pigment clumping, which are all

suggestive of RPE damage.^{14–15} In support of the idea that PDT may affect RPE cells, laboratory studies^{16–17} have shown that there is a change not only in the CNV but also in the RPE cells after PDT. These findings suggest that PDT may affect RPE cells surrounding CNV by inducing cell death, temporary dysfunction and, possibly, angiogenic responses.

PDT exerts its function by photochemical reaction, which is mediated through reactive oxygen species. Considering the previous observations of up regulated vascular endothelial growth factor (VEGF) and down regulated pigment epithelium-derived growth factor (PEDF) expressions in human RPE cells after administration of reactive oxygen species^{18–19} or after exposure to oxidative stresses such as hypoxia–reoxygenation and paraquat,²⁰ together with the observation that triamcinolone acetonide suppressed these angiogenic response in human-cultured RPE exposed to oxidative stress,²⁰ it is possible that PDT-induced oxidative stress may affect VEGF and PEDF expressions in RPE cells and that this PDT-induced proangiogenic effect may be suppressed by triamcinolone acetonide. Thus, in this study, we investigated the expression of these proangiogenic and antiangiogenic factors after PDT, especially focusing on their change in the presence of triamcinolone acetonide.

MATERIALS AND METHODS

Cell line

A human RPE cell line, ARPE-19, was purchased from the American Type Culture Collection (ATCC; Manassas, Virginia,

Abbreviations: ARPE, human retinal pigment epithelial; CNV, choroidal neovascularisation; PDT, photodynamic therapy; PEDF, pigment epithelium-derived factor; RPE, retinal pigment epithelial; RT-PCR, reverse transcriptase-polymerase chain reaction; VEGF, vascular endothelial growth factor

USA). Cell cultures were maintained in Dulbecco's modified Eagle's medium/F-12 (Gibco BRL, Grand Island, New York, USA) supplemented with 10% fetal bovine serum (FBS; Gibco BRL), penicillin (100 U/ml) and streptomycin (100 µg/ml; Gibco BRL), and cultured in a humidified incubator at 37°C in an atmosphere of 5% CO₂. Cells at passages 13–15 were used.

Preparation of verteporfin and triamcinolone acetonide

The contents of a vial of verteporfin (Visudyne, Novartis, Tokyo, Japan) were dissolved in 7 ml distilled water to obtain 2 mg/ml isotonic solution. The solution was further diluted with phosphate-buffered saline at concentrations ranging from 10 to 0.01 µg/ml, and the solution was further diluted by the culture media at the indicated concentrations (5–0.001 µg/ml). Verteporfin at concentrations ranging from 0.1 to 0.001 µg/ml was used to investigate the cell viability and VEGF and PEDF expressions. Triamcinolone acetonide was prepared as described previously²¹ with minor modifications. The contents of a vial (40 mg/ml) of triamcinolone acetonide (Kenakolt-A, Bristol Pharmaceuticals KK, Tokyo, Japan) were diluted with phosphate-buffered saline (PBS) to give a triamcinolone acetonide solution at a concentration of 1 mg/ml, which was diluted with the culture media to give a final concentration of 1 µg/ml. As triamcinolone acetonide was supplied in crystalline suspension, the vial was continually vortexed to ensure dispersion of triamcinolone acetonide particles. The concentration used in the experiment was determined on the basis of previous studies,^{20, 21} and was one tenth of that which showed toxicity for ARPE-19 cells.²¹ The original vial included 9 mg of benzyl alcohol as the preservative, which was diluted in the mixture used in the study to give a final concentration of 0.23 ppm. The concentration was one thousandth of the level presenting no toxic effect on ARPE-19 cells.²¹

Cellular uptake of verteporfin

At 1 h after confluent ARPE-19 cells in 10 cm dishes were incubated with 5 µg/ml of verteporfin, the dishes were rinsed with 10 ml of PBS at least three times. After confirming that there was no detectable level of verteporfin remaining in the final rinsing solutions, 2 ml of radioimmunoprecipitation assay buffer (20 mM Tris-HCl, pH 7.4, sodium dodecyl sulphate 0.1%, Triton X-100 1%, and sodium deoxycholate 1%) was added to each well to lyse the cells. The concentration of verteporfin in the supernatant or in the cell lysate was measured with a spectrophotometer (U-2000A, Hitachi, Tokyo, Japan), where the wavelength for absorbance was set at 440 nm in accordance with previous reports.^{22–23} The measurement was performed in quadruplicate.

Light exposure

Light exposure was performed with a xenon lamp at 0.4 mW/cm² for 5 min. To minimise the effect of thermal damage, the plates were placed on ice during light exposure. Thereafter, the medium was changed to 500 µl of Dulbecco's modified Eagle's medium/F-12 supplemented with 10% FBS with or without 1 µg/ml triamcinolone acetonide and incubated in the dark.

Cell viability analysis

After the treatment, the ARPE-19 cells were incubated in the dark for 2 days. Cell viability was then assessed using two methods: (1) directly counting viable cells with trypan blue for exclusion of dead cells and (2) quantifying mitochondrial dehydrogenase activity using WST-1 colorimetric assay (Roche Diagnostics, Indianapolis, Indiana, USA).

The non-irradiated cells served as controls. Each measurement was performed in quadruplicate.

ELISA

After light exposure, the medium was changed and the cells were incubated in the dark for 2 days. To lyse the cells, 200 µl of RIPA buffer was added to the cells after removing the medium. VEGF protein concentration in the medium and intracellular PEDF protein concentration were measured using the Quantikine Human VEGF Immunoassay ELISA Kit (R&D Systems, Minneapolis, Minnesota, USA) and PEDF ELISA Kit (Chemicon, Pittsburgh, Pennsylvania, USA), respectively, according to the manufacturers' instructions. Absorbance at 450 nm was measured using a microplate reader (Model 3550, Bio-Rad Laboratories, Hercules, California, USA). Protein concentration was normalised by the total protein content, as determined by a bicinchoninic acid protein assay kit (Bio-Rad). Each measurement was performed in quadruplicate.

Real-time reverse transcriptase-polymerase chain reaction

For real-time reverse transcriptase-polymerase chain reaction (RT-PCR), RNA for RT-PCR was isolated using an SV Total RNA Isolation Kit (Promega, Madison, Wisconsin, USA) in accordance with the manufacturer's instructions.

cDNA was prepared using Superscript III for RT-PCR (Invitrogen, USA). Each PCR was carried out in a 20-µl volume using Platinum SYBR Green qPCR SuperMix UDG (Invitrogen) for 15 min at 95°C to denature, followed by 55 cycles of 95°C for 30 s and 60°C for 1 min in Roche LightCycler. Values for each gene were normalised to expression levels of glyceraldehyde-3-phosphate-dehydrogenase. The sequences of the primers used for RT-PCR were as follows: human VEGF, left, 5'-atgtcatggtgatgttga-3'; right, 5'-gcttgctgctgtacctcac-3'; human PEDF left, 5'-tgtgcaggcttagaggact-3'; right, 5'-gttcacgggactttgaaga-3'.

Statistical analysis

The statistical analysis was performed using computer software (JMP V.5, SAS Institute, Cary, North Carolina, USA). The intracellular concentrations of verteporfin were analysed using Student's t test, the cell viabilities by Student's t test with a Bonferroni's correction, the VEGF or PEDF protein level by one-way analysis of variance followed by Dunnett's retrospective analysis test. (Bonferroni-corrected) $p < 0.05$ was considered significant.

RESULTS

Cellular uptake of verteporfin

To test whether triamcinolone acetonide treatment affects cellular uptake of verteporfin, intracellular concentration of verteporfin was measured 1 h after an addition of triamcinolone acetonide. The results showed that the mean (standard deviation (SD)) uptake of verteporfin was approximately 4.15 (0.50) µg/10⁶ cells and 4.30 (0.33) µg/10⁶ cells in the absence and presence of triamcinolone acetonide, respectively, showing that treatment with triamcinolone acetonide does not affect the cellular uptake of verteporfin. The amount of verteporfin uptake in ARPE-19 cells—that is, approximately 4 µg—corresponds to 8% of the total verteporfin administered while in the dishes.

Cell viability analysis

To investigate whether light-activated verteporfin induces cell damage, the number of viable cells was counted and mitochondrial NADH enzyme activity was measured by means of WST-1 analysis as a surrogate marker for cell viability. Verteporfin showed a dose-dependent toxicity on ARPE-19 cells after acute light exposure of 120 mJ/cm² (fig 1A, B). The presence of verteporfin at a concentration of 0.01 µg/ml

affected neither the cell count nor the cell viability as assessed by WST-1 analysis, even after acute exposure to light. On the contrary, almost total cell death was observed after the cells were exposed to verteporfin at a concentration of 0.1 $\mu\text{g/ml}$ and subsequently exposed to light. No significant reduction in the cell count and viability was observed in the non-illuminated cells, even after the cells were exposed to verteporfin to a concentration of 0.1 $\mu\text{g/ml}$. Additionally, the presence of triamcinolone acetonide affected neither the cell counts nor viability, regardless of the light exposure.

Cytokine expression study

The secreted protein levels of VEGF and intracellular levels of PEDF were measured by means of an ELISA assay. Figure 2 shows the results of the ELISA assay of VEGF protein. In the absence of light exposure, the VEGF protein level was not affected by treatment with verteporfin up to a concentration of 0.1 $\mu\text{g/ml}$. After acute light exposure, VEGF protein was significantly higher in the ARPE-19 cells that were exposed to 0.01 $\mu\text{g/ml}$ of verteporfin compared with that secreted after exposure to the vehicle alone ($p < 0.001$), whereas in the presence of verteporfin at a concentration of 0.001 $\mu\text{g/ml}$ the VEGF protein levels were similar to the control. Triamcinolone acetonide inhibited the up regulation of VEGF protein from the ARPE-19 cells exposed to 0.01 $\mu\text{g/ml}$ of verteporfin ($p < 0.001$), reducing the levels to those almost equivalent to the control.

Figure 1 Cell viability of ARPE-19 cells 2 days after photosensitisation with verteporfin (VP). ARPE-19 cells were exposed to 0.01–0.1 $\mu\text{g/ml}$ of verteporfin or vehicle alone for 1 h in the dark, and then illuminated for 15 min. After ARPE-19 cells had been incubated for 2 days, viable ARPE-19 cells were counted (A) and WST-1 cell viability assay was performed (B). During exposure and incubation, 1 $\mu\text{g/ml}$ of triamcinolone acetonide (TA) or vehicle alone was added to the medium. ARPE-19 cells similarly treated, except for illumination, were studied as controls. Cell counts or WST-1 assay results showed dose-dependent toxicity of verteporfin in the ARPE-19 cells illuminated, whereas those without illumination indicated no marked toxicity. Addition of 1 $\mu\text{g/ml}$ triamcinolone acetonide did not cause any significant difference in toxicity. Values indicated are average of quadruplicate experiments, and error bars show SDs.

Figure 3 shows the intracellular PEDF protein levels. At a concentration of 0.001 $\mu\text{g/ml}$, verteporfin did not affect the PEDF protein levels even after light exposure, similar to the results of the VEGF ELISA analysis. After the ARPE-19 cells were exposed to verteporfin at a concentration of 0.01 $\mu\text{g/ml}$, intracellular PEDF protein levels were lower than the control after light exposure ($p = 0.03$). The down regulation of PEDF protein was significantly inhibited by treatment with triamcinolone acetonide ($p = 0.001$).

Real-time RT-PCR was performed to investigate the expression of VEGF and PEDF at mRNA level. The results showed that after acute light exposure in the presence of verteporfin at a concentration of 0.01 mg/ml, the VEGF mRNA levels were higher in the ARPE-19 cells than in the control. Triamcinolone acetonide inhibited the up regulated expression of VEGF. In contrast, PEDF mRNA levels were lower than the control after light exposure in the presence of 0.01 mg/ml verteporfin, and the down regulation of PEDF mRNA was inhibited by treatment with triamcinolone acetonide (fig 4).

DISCUSSION

The results of our study suggest that light irradiation up regulated VEGF and down regulated PEDF in verteporfin-loaded RPE cells at a sublethal dose, which was suppressed by triamcinolone acetonide. VEGF is a major proangiogenic factor with vasopermeable effect, with cumulative evidence suggesting its critical role in CNV induction and in the aggravation of exudative changes caused by CNV.^{24–30} In contrast, PEDF is a potent antiangiogenic factor shown to be secreted by RPE cells,^{31–34} showing marked inhibition of CNV in a murine model.³⁵ Both VEGF and PEDF show strong expression in surgically removed human CNV, owing to age-related macular degeneration,³⁶ suggesting their association with CNV. Together with the above previous studies, our results suggest that the acute progression of exudative retinal detachment¹¹ and temporary increased leakage from CNV³⁷ after PDT is at least partly due to the up regulated VEGF and down regulated PEDF expression in RPE cells. The inhibitory effect of triamcinolone acetonide on the up regulation of VEGF and down regulation of PEDF in RPE cells might be associated with the reduction in the temporary exacerbation of exudative changes shortly after

Figure 2 Vascular endothelial growth factor (VEGF) protein concentration 2 days after photosensitisation with verteporfin (VP). ARPE-19 cells were exposed to 0.001 and 0.01 $\mu\text{g/ml}$ of VP or vehicle alone for 1 h in the dark. After 5 minutes of illumination, the ARPE-19 cells were incubated for 2 days until VEGF protein assay. During the exposure and the incubation, 1 $\mu\text{g/ml}$ of triamcinolone acetonide (TA) or vehicle alone was added to the medium. The ARPE-19 cells similarly treated, except for illumination, were studied as controls. VEGF concentration significantly increased in ARPE-19 cells photosensitised with 0.01 $\mu\text{g/ml}$ of VP without TA ($p < 0.001$), but TA significantly suppressed its expression ($p < 0.001$). Values indicated are the average of quadruplicate experiments, and error bars show SDs. * $p < 0.05$.

Figure 3 Pigment epithelium-derived factor (PEDF) protein concentration 2 days after photosensitisation with verteporfin (VP). ARPE-19 cells were exposed to 0.001 and 0.01 $\mu\text{g/ml}$ of VP or vehicle alone for 1 h in the dark. After 5 minutes of illumination, the ARPE-19 cells were incubated for 2 days. During exposure and incubation, 1 $\mu\text{g/ml}$ of triamcinolone acetate (TA) or vehicle alone was added to the medium. The ARPE-19 cells similarly treated, except for illumination, were studied as controls. PEDF concentration significantly decreased in the ARPE-19 cells photosensitised with 0.01 $\mu\text{g/ml}$ of VP without TA ($p=0.03$), but TA significantly suppressed its reduction ($p=0.001$). Values indicated are the average of quadruplicate experiments, and error bars show SDs. * $p<0.05$.

verteporfin treatment with triamcinolone acetate. This is not due to the difference in the cellular uptake of verteporfin after triamcinolone acetate addition, as cellular uptake was not altered by the addition of triamcinolone acetate. It has been previously reported that treatment with dexamethasone increased PEDF RNA levels in mouse Muller glial cells and C6 rat glioma cells.³⁸ However, further studies are required to elucidate the exact molecular mechanism of how triamcinolone acetate suppresses PDT-induced VEGF up regulation and PEDF down regulation.

Several immunohistochemical studies have focused on the expression of VEGF and PEDF after PDT.^{8, 39} A previous study using human eyes with untreatable malignancy showed that after standard PDT was applied to normal retinobulbar area, the expressions of both VEGF and PEDF were up regulated.⁸ In contrast, another study using surgically excised CNV membranes showed that after PDT was applied to CNV, both increased expression of VEGF and significantly reduced expression of PEDF were observed.³⁹ This and the current study are in good accordance with several previous studies showing that VEGF and PEDF expressions are modulated oppositely by stimuli including hypoxia and oxidative stress.^{18–20} However, considering the inconsistency of the previous studies on the expression of PEDF after PDT,^{8, 39} whether PEDF is up regulated after PDT remains an important issue to be examined in future studies.

The dose-dependent cytotoxicity of triamcinolone acetate has been reported on RPE cells,^{21, 40, 41} leading to concern that triamcinolone acetate might enhance the toxic effect of PDT on RPE. However, cell viability was not affected by the addition of 1 $\mu\text{g/ml}$ of triamcinolone acetate, even after light exposure to verteporfin-loaded cells. This is in good accordance with previous in vitro experiments, which showed that a single administration of triamcinolone acetate was cytotoxic at 10 $\mu\text{g/ml}$ but not at 1 $\mu\text{mol/l}$ (0.43 $\mu\text{g/ml}$) using ARPE-19 cells.^{20, 21}

The present result that triamcinolone acetate suppresses acute proangiogenic response after PDT may justify the coadministration or preadministration of triamcinolone acetate with PDT. However, it should be noted that this study has limitations. Firstly, this study was performed in vitro, using a

Figure 4 Vascular endothelial growth factor (VEGF) and pigment epithelial-derived factor (PEDF) mRNA levels after photosensitisation with verteporfin (VP). ARPE-19 cells were exposed to 0.01 $\mu\text{g/ml}$ of VP 1 h in the dark. After 15 min of illumination, the ARPE-19 cells were incubated for 2 days. During exposure and incubation, 1 $\mu\text{g/ml}$ of triamcinolone acetate (TA) or vehicle alone was added to the medium. The ARPE-19 cells similarly treated, except for illumination, were studied as controls. VEGF mRNA levels were higher and PEDF mRNA levels were lower in the ARPE-19 cells photosensitised with 0.01 $\mu\text{g/ml}$ of VP without TA, but TA suppressed the up regulation of VEGF and down regulation of PEDF. Values indicated are the average of six experiments, and error bars show SDs.

cultured RPE cell line. The effects of triamcinolone acetate on vascular endothelial cells, macrophage and fibrocyte⁴² have not been studied. Additionally, this and other in vitro studies cannot provide any definite information about the concentration of triamcinolone acetate that should be used clinically. Clinically, the intravitreal concentration of the administered triamcinolone acetate at the concentration used in combination treatment with PDT reaches approximately 1000 $\mu\text{g/ml}$,⁴ which is more than 100-fold the dose that may cause RPE damage according to experimental studies. However, in a clinical situation, there has been no triamcinolone acetate-related RPE damage, to the best of our knowledge, suggesting that triamcinolone acetate reaches the RPE layer less efficiently than expected, or that the cytotoxic effect of triamcinolone acetate is ameliorated in an in vivo environment. Therefore, although 1 $\mu\text{g/ml}$ of triamcinolone acetate suppressed the PDT-induced adverse RPE response without affecting RPE cell viability by itself or when used in combination with verteporfin in vitro, the optimum concentration of triamcinolone acetate that should be used clinically remains unknown. Further in vivo experiments and a more detailed clinical analysis are warranted to clarify the effects of PDT and triamcinolone acetate on CNV and to determine the optimum triamcinolone acetate concentration.

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