Toxicity of Triamcinolone Acetonide on Retinal Neurosensory and Pigment Epithelial Cells

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PURPOSE. To study the toxicity of triamcinolone acetonide (Kenalog; Bristol-Meyers Squibb, Princeton, NJ) on retinal pigment epithelial (ARPE-19) and retinal neurosensory (R28) cells.

METHODS. ARPE-19 and R28 were grown in tissue culture in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum. Cells were treated with 50, 100, and 200 μg/mL concentration of triamcinolone acetonide for 2, 6, and 24 hours. The cells were also treated with the steroid without the vehicle and with the vehicle alone, in which triamcinolone acetonide was suspended. Toxicity was determined by trypan blue dye-exclusion and WST-1 mitochondrial dehydrogenase assays.

RESULTS. Vehicle alone did not reduce the viability of ARPE-19 or R28 cells and also did not affect the mitochondrial dehydrogenase activity of the cells. The mean cell viability of ARPE-19 and R28 cells after exposure to triamcinolone acetonide with vehicle 200 μg/mL for 24 hours was 70.7% ± 10.61% and 75.35% ± 12.42%, respectively compared with the untreated ARPE-19 (92.7% ± 6.24%, P < 0.01) and R28 cells (90.63% ± 5.62%, P < 0.001). The mean cell viability of ARPE-19 cells after exposure to triamcinolone acetonide (200 μg/mL) alone without the vehicle was 84.96% ± 0.32%, 85.2% ± 3.26%, and 84.73% ± 2.71% at 2, 6, and 24 hours, respectively, compared with the untreated ARPE-19 cells (P < 0.001). The R28 cells exposed to triamcinolone acetonide (200 μg/mL) without the vehicle also had a significant reduction in the mean cell viability at 24 hours (86.42% ± 3.87%, P < 0.001) and 6 hours (89.03% ± 1.01%, P < 0.01). There was a significant reduction in the mitochondrial dehydrogenase activity in the ARPE-19 cells when treated with both triamcinolone acetonide, with or without the vehicle at a concentration of 200 μg/mL, at all time points (P < 0.01). R28 cells did not have any significant reduction in mitochondrial dehydrogenase activity when treated with triamcinolone acetonide without the vehicle at any of the doses, but there was a significant reduction when the R28 cells were treated with triamcinolone acetonide with vehicle (200 μg/mL) for 24 hours (P < 0.05). Triamcinolone acetonide with vehicle caused a greater reduction in cell viability and mitochondrial dehydrogenase activity than did triamcinolone without vehicle, in both cell lines, although the difference was not statistically significant.

CONCLUSIONS. Triamcinolone acetonide is toxic to proliferating cells of retinal origin in vitro at doses normally used in clinical practice. The vehicle by itself appears to be nontoxic to the cells, but may have a potentiating effect on the cytotoxicity of triamcinolone acetonide. The results of this in vitro study cannot be directly extrapolated to clinical practice, but, based on these data, further studies may be warranted. (Invest Ophthalmol Vis Sci. 2006;47:722–728) DOI:10.1167/iovs.05-0772

Intravitreal triamcinolone acetonide (Kenalog; Bristol-Meyers Squibb, Princeton, NJ) is extensively used to treat macular edema due to diabetic retinopathy,1–3 venous occlusive disease,4 ocular inflammation,5,6 and also cases of choroidal neovascularization (CNV).7–9 The anti-inflammatory potency of triamcinolone acetonide is similar to that of methylprednisolone but significantly less compared with that of dexamethasone.10 The biological half-life of parenterally administered triamcinolone acetonide is 18 to 36 hours,10 whereas the mean elimination half-life in nonvitrectomized eyes is 18.6 days,11 Beer et al.11 found measurable concentrations of triamcinolone acetonide for approximately 3 months after intravitreal injection in nonvitrectomized eyes. The peak aqueous humor concentration ranged from 2.15 to 7.20 μg/mL. The prolonged duration of action of triamcinolone acetonide in the eyes is due to its insoluble form, which causes it to act as a depot injection when injected intravitreally. Dexamethasone is also used clinically to reduce intraocular inflammation, but, because it is only available in a soluble form, its duration of action is much less than that of triamcinolone acetonide. The common adverse effects of ocular steroid therapy are glaucoma and cataract.3,12–15 In addition, given that the commonly used formulation of triamcinolone acetonide (Kenalog; Bristol-Meyers Squibb), is not formulated for the eye, there is a known risk of pseudoendophthalmitis and a hypothetical potential for clinical retinal toxicity from the vehicle when it is injected intravitreally.14–16 There have been reports of toxicity of triamcinolone acetonide on retinal pigment epithelial cells in vitro12,15 whereas ex vivo15 and in vivo14,16 studies have failed to show any significant toxicity in the retina. Triamcinolone acetonide also causes phototoxicity of erythrocytes in vitro and may also have weak photosensitizing properties in vivo.16,17 We performed this study on retinal pigment epithelial (ARPE-19) and neurosensory retinal (R28) cell lines using two different assays to measure the cytotoxicity of triamcinolone acetonide. The toxicity of triamcinolone acetonide suspension with its vehicle, vehicle alone, and the drug alone without the vehicle were studied separately.

MATERIALS AND METHODS

Cell Culture

ARPE-19 cells were obtained from ATCC (Manassas, VA). Cells were grown in 1:1 mixture (vol/vol) of Dulbecco’s modified Eagle’s and Ham’s nutrient mixture F-12 medium (DMEM F-12; Invitrogen-Gibco, Carlsbad, CA), nonessential amino acids 10 mM 1X, 0.37% sodium
bicarbonate, 0.058% l-glutamine, 10% fetal bovine serum, and antibiotics (penicillin G 100 U/mL, streptomycin sulfate 0.1 mg/mL, gentamicin 10 µg/mL, amphotericin B 2.5 µg/mL). R28 cells, which are rat embryonic precursor neurosensory retinal cells, were derived from postnatal day 6 rat retina in the laboratory of one of the authors (GMS).23 R28 cells express genes characteristic of neurons,24 as well as functional neuronal properties.25 R28 cells were cultured in Dulbecco’s modified Eagle’s medium, high glucose (DMEM high glucose; Invitrogen-Gibco) with 10% fetal bovine serum, 1× minimum essential medium (MEM), 10 mM 1× nonessential amino acids, 0.37% sodium bicarbonate, and 10 µg/mL gentamicin.

The cells were plated onto 96-well ELISA plates or 35-mm tissue culture dishes and incubated at 37°C in 5% CO₂ to reach 70% to 80% confluence before being exposed to the drug.

**Exposure to Triamcinolone Acetonide**

Cells were treated with 50, 100, and 200 µg/mL concentration of triamcinolone acetonide (Kenalog; Bristol Meyers Squibb) for 2, 6, and 24 hours. ARPE-19 and R28 cells were also treated with the steroid alone, resuspended in the culture medium at the same concentrations, after removal by centrifugation of the vehicle used for the suspension of triamcinolone acetonide. Triamcinolone acetonide was centrifuged at 5000 rpm for 45 seconds, and the supernatant containing the vehicle of triamcinolone acetonide was resuspended in the culture medium at the corresponding concentrations (50, 100, and 200 µg/mL). The cells were also exposed to the highest concentration of the vehicle that was removed after centrifugation of 200 µg/mL triamcinolone acetonide.

**Cell Viability Assay**

Cell viability assay was performed as previously described.26 Briefly, cells were harvested from the 35-mm dishes by treatment with 0.2% trypsin-EDTA and incubating them at 37°C for 5 minutes. The cells were centrifuged at 1000 rpm for 1 minute and then resuspended in 1 mL of culture medium. Automated cell viability analysis was performed (ViCell analyzer; Beckman Coulter Inc., Fullerton, CA). The analyzer performs an automated trypan blue dye-exclusion assay and gives the percentage viability of cells.

**Mitochondrial Dehydrogenase Assay**

To assess mitochondrial function, mitochondrial dehydrogenase (succinate-tetrazolium-reductase) activity was determined with the WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzenedisulfonate) colorimetric assay (Roche Diagnostics, Indianapolis, IN), as previously described.26 WST-1 is a tetrazolium dye containing an electron coupling reagent that is cleaved by the mitochondrial dehydrogenase enzyme to a formazan dye, and this reaction directly correlates with the number of metabolically active cells. Ten microliters of the formazan dye was added to each well containing cells and medium and incubated for 2 hours at 37°C. Absorbance was measured at 490 nm on a multiwell spectrophotometer (Perkin Elmer, Wellesley, MA). Control wells with culture medium and different concentrations of triamcinolone acetonide but without any cells were also studied, since triamcinolone acetonide is opaque, and as a result influences the spectrophotometer reading. The colorimetric values from these wells were subtracted from the values obtained from the wells containing the cells treated with corresponding concentrations of triamcinolone acetonide to derive the actual colorimetric value. The data presented are from three separate experiments.

**Statistical Analysis**

Data were subjected to statistical analysis by ANOVA (Prism, ver. 3.0; GraphPad Software Inc., San Diego, CA). Newman-Keuls multiple-comparison test was done to compare the data within each experiment. P < 0.05 was considered statistically significant. Error bars in the graphs represent SEM with experiments performed in triplicate.

**RESULTS**

**Dye-Exclusion Assay**

**ARPE-19 Cells.** Vehicle alone did not significantly reduce the viability of ARPE-19 cells (92.03% ± 4.99%). The mean viability of ARPE-19 cells after exposure to triamcinolone acetonide with vehicle 200 µg/mL for 24 hours was reduced significantly (70.7% ± 10.61%), compared with the untreated ARPE-19 cells (92.7% ± 6.24%, P < 0.01). The mean viability of cells exposed to 200 µg/mL triamcinolone acetonide with vehicle for 2 and 6 hours was less than that of the untreated cells, but the difference was not significant (Fig. 1A; Table 1). Also, triamcinolone acetonide with vehicle at doses of 50 and 100 µg/mL did not significantly affect the cell viability at any of the time points. The mean cell viability of ARPE-19 cells after exposure to triamcinolone acetonide (200 µg/mL) alone without the vehicle was 84.96% ± 0.32%, 85.2% ± 3.26%, and 84.75% ± 7.21% at 2, 6, and 24 hours, respectively, compared with the untreated ARPE-19 cells (P < 0.001). Cells exposed to 100 µg/mL of triamcinolone acetonide without the vehicle also had a significant reduction in cell viability at all three time points, whereas cells exposed to 50 µg/mL did not have any significant reduction in viability compared with the untreated ARPE-19 cells (Fig. 1B, Table 2).

**R28 Cells.** R28 cells demonstrated a significant reduction in cell viability after treatment with triamcinolone acetonide with vehicle at 200 µg/mL for 24 hours and 6 hours with a mean cell viability of 75.5% ± 12.42% (P < 0.001) and 31.9% ± 9.49% (P < 0.05), respectively, compared with the untreated R28 cells (90.63% ± 5.62%), whereas exposure for 2 hours did not cause significant reduction in cell viability (Fig. 2A). Lower concentrations of the drug with vehicle did not significantly reduce the cell viability at any of the time points (Table 3). Cells exposed to vehicle alone did not have any significant reduction in cell viability (90.93% ± 5.49%). R28 cells exposed to triamcinolone acetonide (200 µg/mL) without the vehicle also had a significant reduction in the mean cell viability at 24 hours (83.95% ± 3.87%, P < 0.001) and 6 hours (89.03% ± 1.01%, P < 0.01). R28 cells exposed to the lower concentrations of the drug did not have any significant reduction in cell viability compared with the untreated cells (95.7% ± 2.65%, Fig. 2B, Table 4).

The viability of both ARPE-19 and R28 cells was less when the cells were treated with triamcinolone acetonide with vehicle than when treated with triamcinolone without vehicle at the corresponding dosage and time points, although the difference was not statistically significant.

**Mitochondrial Dehydrogenase Assay**

**ARPE-19 Cells.** The mean mitochondrial dehydrogenase activity of ARPE-19 cells treated with 200 µg/mL triamcinolone acetonide with vehicle for 24 hours was significantly less than the activity of the untreated cells (absorbance at 490 nm 0.112 ± 0.074 vs. 0.541 ± 0.016, respectively; P < 0.01). There was also a significant reduction in the mitochondrial dehydrogenase activity of ARPE-19 cells when treated with 200 µg/mL triamcinolone acetonide with vehicle for 2 and 6 hours (P < 0.01, Fig. 3A). The reduction in the enzyme activity of the cells when treated with 50 and 100 µg/mL triamcinolone acetonide with vehicle at any of the time points was not significant. The mitochondrial dehydrogenase activity of cells treated with vehicle alone was 0.488 ± 0.092, which was not significantly different from the untreated ARPE-19 cells (0.541 ± 0.016).

The mean mitochondrial dehydrogenase activity of ARPE-19 cells treated with 200 µg/mL triamcinolone acetonide without the vehicle for 24 hours was 0.017 ± 0.146, which was significantly less than the activity in the untreated cells (0.434 ±
There was also a significant reduction in the mitochondrial dehydrogenase activity when the cells were treated with 200 μg/mL triamcinolone acetonide without the vehicle for 2 and 6 hours (Fig. 3B). ARPE-19 cells treated with 50 and 100 μg/mL triamcinolone acetonide without the vehicle did not have any significant reduction in mitochondrial dehydrogenase activity.

R28 Cells. R28 cells treated with 200 μg/mL triamcinolone acetonide with vehicle for 24 hours had a mean mitochondrial dehydrogenase activity of 0.018 ± 0.039, which was significantly less than the mean activity in the untreated R28 cells (0.271 ± 0.016; P < 0.05). Cells treated with 100 and 50 μg/mL triamcinolone acetonide with vehicle for 24 hours had a mean mitochondrial dehydrogenase activity of 0.079 ± 0.061 and 0.080 ± 0.114, respectively, which was not significantly less than the activity in the untreated R28 cells (P > 0.05). Cells treated with triamcinolone acetonide for 2 and 6 hours also did not have any significant reduction in mitochondrial dehydrogenase activity. R28 cells treated with vehicle alone did not have any significant reduction in mitochondrial dehydrogenase activity (0.239 ± 0.034) compared with the untreated cells (P < 0.05). Cells treated with triamcinolone acetonide without the vehicle did not have any significant reduction in their mean mitochondrial dehydrogenase activity at any of the three concentrations and time points (Fig. 4B).

As with the dye-exclusion assay, the mitochondrial dehydrogenase activity was higher in both the ARPE-19 and R28

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**Table 1. Viability of ARPE-19 Cells Treated with Triamcinolone with Vehicle**

<table>
<thead>
<tr>
<th>Concentration (μg/mL)</th>
<th>2 h</th>
<th>6 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>—</td>
<td>—</td>
<td>92.7 ± 6.24</td>
</tr>
<tr>
<td>50</td>
<td>90.66 ± 4.25</td>
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<td>76.86 ± 10.75</td>
<td>70.7 ± 10.61*</td>
</tr>
<tr>
<td>Vehicle</td>
<td>—</td>
<td>—</td>
<td>92.05 ± 4.99</td>
</tr>
</tbody>
</table>

* Significant (P < 0.05).

**Table 2. Viability of ARPE-19 Cells Treated with Triamcinolone without Vehicle**

<table>
<thead>
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<th>Concentration (μg/mL)</th>
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<th>6 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
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<td>—</td>
<td>—</td>
<td>96.46 ± 3.85</td>
</tr>
<tr>
<td>50</td>
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<td>93.3 ± 1.99</td>
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<tr>
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<td>88.43 ± 0.37*</td>
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<tr>
<td>200</td>
<td>84.96 ± 0.32*</td>
<td>85.2 ± 3.26*</td>
<td>84.73 ± 2.71*</td>
</tr>
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</table>

* Significant (P < 0.05).
cells when treated with triamcinolone acetonide without the vehicle compared with cells treated with triamcinolone acetonide with vehicle at the corresponding doses and time points, but this was not statistically significant.

**DISCUSSION**

We found in our study that 200 μg/mL triamcinolone acetonide with or without the vehicle was toxic to both retinal cell lines in vitro using both the cell viability and mitochondrial dehydrogenase assays, but vehicle alone was not toxic to the cells. The usual clinical dosage of intravitreal triamcinolone acetonide is 4 mg, and assuming that the vitreous volume is 4 mL, the intravitreal concentration of triamcinolone acetonide should be 1 mg/mL. We had performed experiments on R28 and ARPE-19 cells with 1 mg/mL triamcinolone acetonide, but this decreased the cell viability to <10% at 24 hours' exposure (data not shown), and hence we designed the study with 50-, 100-, and 200-μg/mL concentrations. Because triamcinolone acetonide is a heavy depot formulated suspension, it settles in the inferior vitreous cavity. Whereas there is certainly distribution of the drug throughout the vitreous cavity due to diffusion and constant eye movements, it is possible that the drug does not distribute equally in the vitreous cavity and that the concentration of the drug at the macula is different (presumably lower) than in the inferior retinal periphery. However, when triamcinolone acetonide is used as a surgical adjuvant to identify...
tify the cortical vitreous and posterior hyaloid during vitrectomy surgery, including macular hole surgery, the triamcinolone crystals come in direct contact with the neurosensory retina, and in the case of macular holes and retina tears, with the retinal pigment epithelium. In this situation, the results of this study suggest that direct contact of triamcinolone crystals with the retina (particularly the retinal pigment epithelium) may result in increased toxicity to the retinal cells.

Triamcinolone acetonide has been shown to be toxic to retinal pigment epithelial cells in vitro, whereas ex vivo and in vivo studies have failed to show any significant toxicity to the retina. Yeung et al. reported that triamcinolone acetonide with the vehicle was toxic to ARPE-19 cells and human glial cells (SVG cells) by the mitochondrial dehydrogenase (MTT) assay, but the effect of triamcinolone acetonide without the vehicle was not studied. They studied three concentrations of 10, 100, and 1000 μg/mL over days 1, 3, and 5, and found that there was significant reduction in the mitochondrial dehydrogenase activity of SVG cells at 24 hours at 100 and 1000 μg/mL. They did not find a significant reduction in the absorbance of ARPE-19 cells at 24 hours with any of the concentrations. In contrast, we found in our study that ARPE-19 cells had a significant reduction in the absorbance of ARPE-19 cells at 24 hours with any of the concentrations. In contrast, we found in our study that ARPE-19 cells had a significant reduction in the mitochondrial dehydrogenase activity when exposed to 200 μg/mL triamcinolone acetonide with the vehicle for even 2 hours. In addition, we found that 200 μg/mL triamcinolone acetonide without the vehicle also caused a significant reduction in the enzyme activity at 24 hours. We also performed the cell viability assay in our study, and triamcinolone acetonide without the vehicle was toxic to ARPE-19 cells at concentrations of 100 and 200 μg/mL at all three time points, including the earliest time point of 2 hours. Similarly, we found that 200 μg/mL triamcinolone acetonide, with or without the vehicle, was toxic to R28 cells according to the dye-exclusion assay.

Citing concerns about the toxicity of the preservative used in triamcinolone (Kenalog; Bristol-Meyers Squibb), Bakri and Beer in a small retrospective, noncomparative series showed that preservative free triamcinolone acetonide was nontoxic to the retina. They used a formulation that did not contain benzyl alcohol as a preservative, but used a vehicle containing polysorbate 80, dibasic and monobasic sodium phosphate, polyglycol, and sodium chloride. Hida et al. showed that the vehicle (including the preservative) of triamcinolone acetonide was nontoxic to the retina of rabbits even when injected in double strength, although the vehicles of betamethasone sodium phosphate, methylprednisolone acetate, dexamethasone acetate, and sodium phosphate caused retinal damage. We found in our study by two different assays that the vehicle itself was nontoxic to both ARPE-19 and R28 cells. This finding is in agreement with the results reported by Yeung et al. Also, in our study, triamcinolone acetonide with vehicle showed a trend of being more cytotoxic than did triamcinolone acetonide without the vehicle, although the difference was not statistically significant.

This study shows that triamcinolone acetonide in the Kenalog formulation (Bristol-Meyers Squibb) is toxic to retinal cells at various concentrations at short exposure times, whereas the vehicle including the preservative benzyl alcohol is not toxic. However, the observation that triamcinolone without the vehicle was less toxic than triamcinolone with the vehicle suggests that the vehicle may have a potentiating effect on the toxicity of triamcinolone acetone, although the mechanism is unknown. The concentrations used in this study cannot be directly extrapolated to clinical practice, as this study...
was performed in vitro, and also because the steroid does not distribute evenly in the vitreous gel due to its depot formulation. In addition, the cells used were still capable of proliferation, which is different from normal clinical conditions and may limit the interpretation of the results. Regardless, the results of this study suggest that clinically used doses of triamcinolone acetonide, with or without the vehicle may cause cytotoxicity in retinal neurosensory and pigment epithelial cells, potentially blunting the clinically observed benefit of triamcinolone acetonide.

References


