Triamcinolone Acetonide-Mediated Oxidative Injury in Retinal Cell Culture: Comparison with Dexamethasone

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PURPOSE. To investigate the cytotoxicity of triamcinolone acetonide (TA) and dexamethasone (DXM) in various types of cells in retinal cell culture.

METHODS. Primary rat retinal cell cultures were treated with 25 to 800 µg/mL TA (58 µM–1.8 mM) or DXM (48 µM–1.6 mM) for 12 to 24 hours. Cell survival and death were assessed chemically by measuring cellular DNA contents using DNA-binding fluorescent dye and morphologically by propidium iodide staining. Standard methods were used for immunocytochemistry, immunoblots, and ELISA measurements. Retinal cellular oxidative stress was measured under a fluorescence microscope using 5-(and-6)-carboxy-2′,7′-difluorodihydrofluorescein diacetate. Changes in the level of several antioxidative proteins were investigated using immunoblots.

RESULTS. Exposure to 100 to 800 µg/mL TA (0.23–1.8 mM) or 800 µg/mL DXM (1.6 mM) for 24 hours caused a significant reduction in the number of retinal cells in culture, in a glucocorticoid receptor-independent manner. Of cell types in retinal cell cultures, astrocytes were most sensitive to TA and DXM. TA-induced cytotoxicity was mediated by oxidative stress. p38 kinase, c-Jun N-terminal kinase (JNK), caspase-1, and caspase-3 were involved in oxidative injury by TA. In addition, levels of antioxidative proteins increased after TA exposure.

CONCLUSIONS. TA induces oxidative injury to cultured retinal cells in a glucocorticoid receptor-independent manner. These results suggest that TA has a significantly higher toxic potential in retinal cell culture than more water-soluble DXM. For long-term anti-inflammatory effects, devices that are designed for the sustained release of water-soluble steroids may be safer. (Invest Ophthalmol Vis Sci. 2007;48:5742–5749) DOI:10.1167/iovs.07-0566

Triamcinolone acetonide (TA) is an intermediate-acting, crystalline corticosteroid that has been used to treat inflammatory ocular diseases. TA is typically administered by periocular injection, though in recent studies TA has been administered by intravitreal injection for the treatment of macular edema caused by diabetic retinopathy,1,2 retinal vein occlusion,3 proliferative vitreoretinopathy,4 and exudative age-related macular degeneration (AMD).5 Administration of TA by intravitreal injection was shown to result in higher intravitreal TA concentrations than when the drug was administered by sub-Tenon injection. In addition, intravitreal injection of TA has been shown to maintain drug concentrations for a longer period.6,7 Although TA has a weaker anti-inflammatory potency than dexamethasone (DXM), it has the advantage of existing in a sustained-release crystalline form and thus is used preferentially for local depot injection.

Although TA shows desirable anti-inflammatory effects, several studies have raised concerns about the possible cytotoxicity of intravitreal TA injections. For instance, Yeung et al.8 recently reported that TA has a greater toxic potential in a cultured retinal pigment epithelium (RPE) cell line than DXM and hydrocortisone. More recently, Kuppermann et al.9 demonstrated that TA causes the death of retinal neurosensory and pigment epithelial cells.

Considering the widespread use of intravitreal TA injections in various ocular conditions, elucidating the cytotoxic potential and mechanism of action of TA is clinically important. Therefore, we compared the toxic effects of TA and DXM on various types of cultured retinal cells. In addition, we sought to evaluate the possible mechanisms that contribute to TA-mediated cytotoxicity.

MATERIALS AND METHODS

Primary Retinal Cell Culture

Newborn Sprague-Dawley rats were handled in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Primary cell cultures, including neurons, astrocytes, and photoreceptor cells, were generated from the retinas of newborn (postnatal day 1 or 2) Sprague-Dawley rats, as previously described.10 Briefly, retinas were isolated, placed in Hanks balanced salt solution (Gibco, Grand Island, NY) lacking Ca2+ and Mg2+ and mechanically dissociated into single cells by trituration with fire-polished Pasteur pipettes. Dissociated cells were plated on poly-l-lysine-coated 24-well plates (three retinas per plate). The plating medium was based on Eagle minimum essential medium (MEM; Gibco) supplemented with 10% heat-inactivated fetal bovine serum (HIFBS) and 25 mM KCl. Retinal cultures were maintained at 37°C in a humidified 5% CO2 incubator and were used in experiments after 10-day culture in vitro.

EXPOSURE TO DRUGS

Before drug exposure, the preexisting medium was washed out three times with serum-free medium (Eagle MEM, Earle salts, supplemented with human albumin and glucose-free). Then TA (Kenalog; Bristol Meyers Squibb, Princeton, NJ), DXM (dexamethasone sodium phosphate; Yuhan, Chungwon, Korea), or another drug was added to the retinal cell culture in serum-free medium for the indicated time points.

ASSESSMENT OF CELL SURVIVAL AND DEATH

For most experiments, survival of the retinal cell was quantitatively assessed using a cell proliferation assay kit (CyQuant NF Cell Proliferation Assay Kit; Invitrogen).
ation Assay Kit; Molecular Probes, Eugene, OR) according to the manufacturer’s protocol. This assay is based on measurement of cellular DNA contents by fluorescent dye binding. The extent of proliferation was determined by comparing cell counts for samples treated with drugs with untreated controls. Fluorescent intensity was measured using a fluorescence microplate reader (SpectraMax; Molecular Devices, Union City, CA) with excitation at approximately 485 nm and emission detection at approximately 530 nm.

Propidium iodide (PI; Sigma-Aldrich, St. Louis, MO), a membrane-impermeant nuclear dye that enters only dead cells, was used to detect cell death in mixed retinal cultures in addition to a cell proliferation assay kit. PI (5 μg/mL) was added to the medium, and cell membrane disruption was assessed by fluorescence microscopy (IX70; Exciter filter BP510–550/Barrier filter BA590; Olympus, Tokyo, Japan). Dead cells were counted in five randomly chosen ×200 fields.

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde for 1 hour at room temperature and were permeabilized with 0.2% Triton X-100 for 10 minutes. After blocking with 2% bovine serum albumin, fixed cells were incubated overnight at 4°C with the following primary antibodies: anti–MAP-2 (1/500; Sigma), anti–GFAP (1/1000; Santa Cruz Biotechnology, Santa Cruz, CA), anti–Thy-1 (1/500; Santa Cruz), and anti–γ-
aminobutyric acid (GABA; 1/1000; Sigma). The cultures were then treated with a fluorescence-conjugated secondary antibody (Alexa Fluor 488 donkey anti-rabbit IgG; 1:1000; Molecular Probes, Leiden, Netherlands) for 2 hours at room temperature. For negative controls, cultures were treated only with secondary antibody. Immunoreactive neurons or astrocytes were counted as surviving cells if they exhibited intact cell body and proximal neurite morphology, whereas those with damaged cell bodies or disrupted cell membranes as well as obvious cellular debris were excluded. The number of surviving cells was counted in five randomly chosen ×200 fields in each well.

Measurement of Reactive Oxygen Species in Cells

Levels of cellular reactive oxygen species were measured using the fluorescent probe 5-(and-6)-carboxy-2',7'-dihydrodityridofluorescein diacetate (carboxy-H2DFFDA, Molecular Probes, Eugene, OR). In brief, cells were incubated with 10 μM carboxy-H2DFFDA for 10 minutes at 37°C in the dark and then washed in MEM free of phenol red (Earle salts, supplied glutamine free). Cells were observed under the fluorescence microscope and photographed. Care was taken to obtain photographs precisely at 20 seconds after the illumination onset.

Assay of Caspase-1 and Caspase-3 Activity

The caspase-1 fluorometric protease assay kit, YVAD (Chemicon, Temecula, CA), and the caspase-3 detection kit (FITC-DEVDFMK; Calbiochem, San Diego, CA) were used to detect caspase-1 and caspase-3 activity, respectively. Experiments were conducted according to the manufacturer’s protocols.

Immunoblots for the Mitogen-Activated Protein Kinase Family, Peroxiredoxin-1 and -2, Cu/Zn SOD, and Mn SOD

After they were washed with serum-free medium, cells were suspended in lysis buffer containing 20 mM Tris-Cl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 μM Na3VO4, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride and were centrifuged. Equal amounts of proteins were loaded and electrophoresed on a 10% sodium dodecyl sulfate-polyacrylamide gel and then transferred to poly-vinylidene difluoride membrane (PVDF; Millipore, Bedford, MA). The membrane was blocked with 5% nonfat dried milk for 1 hour and incubated overnight at 4°C with the following antibodies: anti-JNK, anti-phospho-JNK (p-JNK), anti-p38, anti-p-p38, anti-extracellular signal-regulated kinase-1/2 (anti-Erk-1/2), anti-p-Erk-1/2 (all Santa Cruz), anti-peroxiredoxin-1 and anti-peroxiredoxin-2 (LabFrontier, Seoul, Korea), anti-Cu/Zn superoxide dismutase (SOD) (StressGen, Victoria, BC Canada), or anti-Mn SOD (StressGen) antibodies. The secondary antibody was a goat anti-rabbit or anti-mouse IgG (Amersham Life Science, Piscataway, NJ) conjugated to horseradish peroxidase. A chemiluminescence substrate (Pierce Biotechnology, Rockford, IL) was used to visualize the immunoreactive proteins.

Drugs and Chemicals

The pan-caspase inhibitor (z-VAD(Ome)-FMK), caspase-1 inhibitor (Ac-AVAALLPVALLAP-YVAD-CHO), and caspase-3 inhibitors (z-D(Ome)E(Ome)V(DOme)-FMK), 2-amino-3-methoxyflavone (PD98059, inhibitor of Erk-1/2), 1,4-diamino-2,3-dicyano-1,4-bis[2-aminoanthi-lo] butadine (U0126, inhibitor of Erk-1/2), 4-(4-fluorophenyl)-2-(4-methylthiophenyl)5-(4-pyridyl)-1H-imidazole (SB203580, inhibitor of p38), and anhtra[1-9c]pyrazol-6(2H)-one (SP600125, inhibitor of JNK) were purchased from Calbiochem (San Diego, CA). Cycloheximide (protein synthesis inhibitor), RU-486 (mifepristone), 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo(F)quinoxaline (NBQX; glutamate receptor antagonist), 4-(2-aminoethyl) benzenesulfonylfluoride (AEBSF; selective inhibitor of NADPH oxidase), catalase, trolox (antioxidant), and benzyl alcohol were purchased from Sigma (St. Louis, MO). Brain-derived neurotrophic factor (BDNF; a member of neurotrophin family, which shows potent antiapoptosis effects in many cells of neuronal origin) and epidermal growth factor (EGF) were obtained from R&D Systems (Minneapolis, MN), and dizocilpine maleate (MK-801; glutamate receptor antagonist) was purchased from Tocris Cookson (Ellisville, MO).

Statistical Analysis

All data are presented as mean ± SD. Two-tailed t-test with Bonferroni correction for appropriate multiple comparisons was performed. P < 0.05 was considered to represent a significant difference.

RESULTS

The treatment of retinal cells with TA (400 μg/mL) for 24 hours was highly toxic, as evidenced by numerous dead cells shown by propidium iodide (PI) staining (Fig. 1a). In contrast, little damage was observed when cells were treated with the same concentration of DXM compared with sham-washed controls. To determine the concentration-toxicity relationship between TA and DXM in retinal cells, cells were exposed to TA 25 to 800 μg/mL (58 μM–1.8 mM) or DXM 25 to 800 μg/mL (48 μM–1.6 mM) for 24 hours. As shown in Figures 1B and 1C, TA treatment for 24 hours showed significant toxicity at 100 μg/mL (0.23 mM) concentration or higher. In contrast, DXM was less toxic, showing significant yet modest cytotoxicity only at the higher concentration of 800 μg/mL (1.6 mM). The vehicle for TA (benzyl alcohol) alone did not affect the viability of cultured retinal cells (data not shown), even at the highest concentration used (0.0198% in 800 μg/mL TA).

To test whether the glucocorticoid receptor mediates the cytotoxicity of TA or DXM, retinal cells were exposed to 400 or 800 μg/mL TA or 800 μg/mL DXM, for 24 hours, either alone or with 5 μM of the selective glucocorticoid receptor antagonist, mifepristone (RU486; Fig. 2). The toxicity induced by 400 μg/mL, 800 μg/mL TA, or 800 μg/mL DXM was not significantly attenuated by mifepristone. Hence, the glucocorticoid receptor is unlikely to significantly contribute to TA- or DXM-mediated retinal cell toxicity in culture.

Retinal cell culture contains most kinds of retinal cells. To determine whether certain types of cells are more sensitive to TA cytotoxicity than others, immunocytochemical staining for GFAP (astrocytic marker), MAP-2 (general neuronal marker),...
Thy-1 (ganglion neuronal marker), or GABA (GABAergic neuronal marker) was performed. As shown in Figures 3A and 3B, TA exposure markedly and selectively reduced the number of GFAP-positive cells, whereas the number of MAP2- and GABA-positive neurons did not decrease. Thy-1-positive ganglion neurons, on the other hand, exhibited intermediate sensitivity to TA. In contrast, none of the cell types tested appeared damaged in response to 24-hour DXM treatment (not shown).

The mechanism of TA-mediated cytotoxicity in the retina is largely unknown. We therefore examined the oxidative injury pathway as a possible mechanism for TA-induced cytotoxicity. Our results indicate that the level of reactive oxygen species increased within 6 hours of 400 μg/mL TA treatment of retinal cell cultures when compared with sham-washed controls; no change in DCF fluorescence was noted in the latter (Fig. 4A). In contrast, the same concentration of DXM had no such effect. In addition, levels of four antioxidant enzymes, peroxiredoxin-1 and -2, Cu/Zn SOD, and Mn SOD, increased in the retina after TA exposure (Fig. 4B).

Consistent with the major role of oxidative stress in TA cytotoxicity, TA-induced cell death was substantially reduced by antioxidant compounds such as trolox, AEBSF, and catalase (Fig. 4C). On the other hand, addition of the glutamate receptor antagonists MK-801 and NBQX did not attenuate retinal cytotoxicity. In addition, compounds that reduce apoptosis in certain systems, such as cycloheximide, BDNF, and EGF, did not protect against TA-induced cytotoxicity.

Although oxidative stress can result in cell death through diverse intracellular events, stress-activated kinases have been demonstrated to play significant roles in many cases. To determine whether these kinases are activated by TA treatment, Western blot analysis was performed. The level of phosphorylation of p38, JNK, and Erk was increased after 3 to 12 hours of TA treatment (Fig. 5A). Consistent with the kinase activation profile, inhibitors of p38 and JNK attenuated TA-induced cell

**FIGURE 3.** TA neurotoxicity on different retinal cell types. (A) Fluorescence photomicrographs of retinal cell cultures immunostained for GFAP (Aa, Ab), MAP2 (Ac, Ad), Thy-1 (Ac, Af), and GABA (Ag, Ah) protein. Cultures were sham washed (Aa, Ac, Ae, Ag) or underwent 12-hour exposure to 400 μg/mL TA (Ab, Ad, Af, Ah). Scale bar, 100 μm. (B) Bars denote number of surviving GFAP-positive, MAP2-positive, Thy-1-positive, and GABA-positive cells of five randomly chosen ×200 fields (n = 5) in sham-washed control cultures and in cultures treated for 12 hours with 400 μg/mL TA (*P < 0.05, difference from controls).
death, whereas Erk inhibitors such as PD98059 and U0126 had no protective effect (Fig. 5B).

Although oxidative stress can cause overt necrotic cell death, many studies have demonstrated that it can cause caspase-dependent apoptosis. To examine whether caspasess contribute to TA-mediated cytotoxicity, selective inhibitors of caspase-1 and caspase-3, as well as a broad-spectrum caspase inhibitor, were tested against TA-induced cytotoxicity. Our results indicate that all three inhibitors substantially attenuate TA-induced cytotoxicity (Fig. 6A). These results are consistent with activity assays showing that both caspase-1 (Fig. 6B) and caspase-3 (Fig. 6C) were activated in response to TA. Nontoxic DXM exposure, in contrast, did not activate either caspase.

Finally, we examined whether oxidative stress plays a causative role in caspase activation. To address this issue, the antioxidants mentioned were used and were shown to be effective in reducing TA-mediated cytotoxicity. All three antioxidants tested (Trolox, AEBSF, and catalase) inhibited the activation of TA-induced caspase-1 and caspase-3 (Figs. 7A, 7B, respectively). Consistent with other studies, our results showed that oxidative stress is a key upstream event in TA-induced caspase activation.

**DISCUSSION**

Although some studies have reported TA-induced cytotoxicity, this subject remains controversial. The present study showed that exposure of cultured retinal cells to TA, at concentrations achievable in the eye after local injection, causes cell death within 12 to 24 hours. Given that the solvent...
for TA does not cause cytotoxicity, it is likely that TA has an intrinsic cytotoxic effect in its current crystalline form. Although we acknowledge that in vitro studies should be interpreted with caution, our results clearly suggest that care should be exercised when using TA as a local anti-inflammatory agent in the eye.

A notable feature of our study was that TA induces selective death of GFAP-positive astrocytes when compared with the whole neuronal population. Given that astrocytes provide support for the survival of neurons, early astrocytic injury may result in late neuronal death in the intact retina. Among retinal neurons, Thy-1-positive neurons appeared to be more sensitive than other cell types, such as GABA-positive neurons.

**FIGURE 5.** The role of p38 MAPK and JNK in TA-induced retinal cell death. (A) Retinal cell lysates were collected at indicated times after treatment with 400 μg/mL TA and were immunoblotted with anti-phospho-p38 MAPK, anti-p38 MAPK, anti-phospho-JNK, anti-JNK, anti-phospho-Erk1/2, and anti-Erk1/2 antibodies. Although the total levels of p38, JNK, and Erk1/2 did not change, phosphorylated p38, JNK, and Erk1/2 increased after 3 to 12 hours of TA treatment. (B) Bars denote the percentages of surviving cells measured by (n = 9) the cell proliferation assay kit in retinal cultures after 24-hour treatment with 800 μg/mL TA alone (CTL) or with the addition of 50 μM PD98059, 10 μM U0126, 2.0 nM SB203580, or 0.2 nM SP600125. Although the Erk1/2 inhibitors showed little effect, the inhibitors of p38 MAPK and JNK significantly reduced TA-induced cell death.

**FIGURE 6.** The role of caspases in TA-induced retinal cell death. (A) Bars denote the percentages of surviving cells in retinal cell cultures after 24-hour treatment with 800 μg/mL TA alone or with the addition of 1 nM caspase-1 inhibitor, 1 nM caspase-3 inhibitor, or 1 nM pan-caspase inhibitor. All the caspase inhibitors showed significant cytoprotective effects against TA toxicity. Bars represent the activity of caspase-1 (B) or caspase-3 (C) (fluorescence, arbitrary unit; n = 9) in retinal cell cultures, at indicated time points after sham-wash (CTL) or the addition of 400 μg/mL TA or DXM. *P < 0.05.
The mechanism of TA-induced cytotoxicity has not yet been systematically studied, and our results argue against the involvement of glucocorticoid receptors. We therefore examined whether other general injury mechanisms are involved in TA-mediated cytotoxicity. Our data indicate that TA treatment induces an increase in reactive oxygen species levels before overt cell death. Furthermore, different antioxidants are effective in reducing TA-mediated cytotoxicity; hence, oxidative stress may be a key injury mechanism in retinal cells. Further supporting the role of oxidative stress in TA cytotoxicity, several proteins known to be involved in the defense against oxidative stress—peroxiredoxins and SODs—were induced after TA treatment. However, the precise mechanism regarding how TA induces oxidative stress in retinal cells is unknown and may need further investigation.

Because TA tends to precipitate in solution, it seems possible that direct contact of cells with precipitates may have increased local concentrations of TA; in this case, the toxic potency of TA might have been overestimated in comparison with that of DXM. However, because the peak concentration in vivo (4 mg in 4 mL, 9.2 mM) is higher than toxic concentrations in vitro (100–800 μg/mL, 0.23–1.8 mM) and because similar precipitations would also occur in vivo, the current TA toxicity in culture is likely relevant.

Although oxidative injury can cause cell death through diverse intracellular pathways, a number of studies have shown that stress-activated kinases may play key roles. Our results are consistent with this notion and show that p38 and JNK are the predominant kinases implicated in TA-induced oxidative cell death. Caspases are another class of protein that mediate oxidative cell injury in many systems, including retinal cells. Consistent with other studies, our retinal cell cultures showed that the activation of caspase-1 and caspase-3 contributes to TA-induced cell death. Given that antioxidants block caspase activation, it is likely that oxidative stress is an upstream event of caspase activation.

It is also noteworthy that despite its more potent glucocorticoid effects, DXM, a more water-soluble glucocorticoid, has weaker cytotoxic potency than TA. The difference between the cytotoxic potency of TA and of DXM may be attributed, in part, to the difference in their formulations. TA is administered in suspension and may make direct contact with cells, thereby causing local toxicity. This cell–drug contact may have more significant implications in patients who have undergone vitrectomy because eyes are then more prone to the formation of extensive epiretinal deposits. A recent study by Szurman et al. showed that nonadherent, filter-purified TA is nontoxic to ARPE19 cells at concentrations of up to 1000 μg/mL. However, the sustained-release property of TA makes it an attractive therapeutic option. Filtering this compound would thus rid TA of its most desirable property. Alternatively, the use of sustained-release devices in culture would render less toxic soluble corticosteroids such as DXM would be a preferable option in terms of long-term bioavailability of glucocorticoids.

References


