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Different biocompatibility of crystalline triamcinolone deposits on retinal cells in vitro and in vivo☆

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Abstract

Epiretinal deposits of triamcinolone acetonide (TA) can be detrimental to retinal cells in vitro as several laboratory studies have shown. This contrasts with the good clinical experience of intravitreal TA use. We investigated the effect of TA crystals on retinal cells concerning the critical dose range, a potential cell recovery, the drug–tissue interaction and what protective biological factors could explain the discrepancy between in vivo and in vitro results. A human retinal pigment epithelium cell line (ARPE19) and transformed rat retinal ganglion cells (RGC5) were used. Purified TA crystals were either added directly on top of the cell cultures or on top of membrane filter inserts, basement membrane sheets or porcine vitreous with the cells growing underneath. To determine the number of live versus dead cells fluorescent stains were used. Proliferation and viability were measured using the MTT assay and the mean inhibitory dose (ID_{50}) calculated with or without a filter. Cell recovery was measured after transient TA exposure (0.01–1 mg/ml) compared to continuous exposure after 7 days. To exclude a mere mechanical effect of epicellular deposits the TA crystals were replaced by glass pearls in a serum-free medium and the MTT toxicity assay was performed after 24 h. Without direct contact of TA crystals with the cells only a moderate decrease of mitochondrial activity was observed that fully recovered after transient exposure and showed a clinically safe ID_{50} of 7.7 mg/ml. In contrast, direct exposure to even minute crystalline deposits for 7 days caused a rapid progressive and irreversible cell death being significant far below clinically used concentrations (ID_{50} 0.058 mg/ml). Direct exposure to glass pearls did not show any loss of viability. Both basement membrane sheets and vitreous reliably prevented direct cytotoxicity to underlying retinal ganglion cells. Our findings suggest that irreversible TA cytotoxicity in a cell culture setting occurs earlier than previously assumed in the presence of even minute epicellular deposits. But in most clinical situations epiretinal TA deposits seem not to be harmful to ocular cells as protective biological factors may prevent close apposition of TA crystals to susceptible retinal cells. However, in eyes that have undergone vitrectomy with ILM peeling epimacular deposits could be critical.

Keywords: triamcinolone acetonide; epiretinal deposit; retinal cytotoxicity; intravitreal; ocular cell culture

1. Introduction

The intravitreal administration of crystalline triamcinolone acetonide (TA) either alone or in combination with photodynamic therapy remains a common treatment for various ocular disorders (Jonas, 2006). Since the initial report of Machemer et al. (1979) the increasing use of intravitreal TA injection has been accompanied by a controversial discussion on potential adverse effects that are very important in view of the off-label use of the drug. Besides the well-known clinical side-effects (Jaissle et al., 2004) also a direct retinal toxicity of the drug is suspected. Although several studies support the clinical safety (Danis et al., 2000; Jonas et al., 2003), other authors suspected the preservative of commercial TA preparations to

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be toxic (Hida et al., 1986). In consequence, several techniques have been introduced to purify the commercially available formulation and make it preservative-free (Jonas et al., 2000; Nishimura et al., 2003). In a recent laboratory investigation, however, Yeung et al. (2003) comprehensively showed the effect of the preservative to be less important than assumed and demonstrated a pronounced cytotoxicity of the corticosteroid itself through induction of apoptotic cell death. This was confirmed by recent studies that found a loss of cellular viability even at low TA concentrations, mostly around clumped TA particles (Narayanan et al., 2006; Shaikh et al., 2006). These findings raised a debate about the discrepancy between the hitherto good clinical experience and the laboratory evidence for toxicity.

Recently, the observation was made that only the close apposition of crystalline TA particles to the cells, but not the dissolved drug is cytotoxic (Szurman et al., 2006). As the direct adherence of TA crystals to the apical cell surface seems to cause a local, rapid-progressive cytotoxicity, epiretinal deposits of TA must be viewed critically.

The findings of different TA toxicity depending on direct adherence to cells or not might in part explain the discrepant observation on in vivo and in vitro toxicity. But still several questions remain unanswered. First, a safe threshold dose should be determined for both adhering and non-adhering TA, if to be used clinically with confidence. Furthermore, it is important to consider the specific in vivo situation. As epiretinal deposits often resolve within a few days it might be important if the toxic effects of sedimented TA are permanent or reversible. Recovery of cells after transient exposure to a toxic agent could explain the clinically observed biocompatibility of TA. On the other hand, a potential recovery would be contradictory to the fast drug–tissue interaction that has been previously reported in the presence of cell-adhering TA crystals (Szurman et al., 2006). The underlying mechanism has not been conclusively defined yet. The rapid alteration of cellular morphology observed could be either due to a mechanical disruption of the membrane integrity or a drug-related chemical toxicity of TA crystals. Discriminating these effects could be a key issue to understand the different biological responses to TA crystals in vivo and in vitro. Finally, one should take into account the specific conditions given at the vitreoretinal interface, where biological structures could impede direct contact of TA and hence prevent cytotoxicity to susceptible retinal cells. Therefore, it is worthwhile defining the protective properties of biological factors like vitreous or inner limiting membrane (ILM) or conversely, if absent the enhanced susceptibility to TA-induced toxicity.

2. Material and methods

2.1. Cell culture

The rat ganglion cell line (RGC5) was kindly provided by Professor Neeraj Agarwal (UNT Health Science Center, Fort Worth, TX) and the ARPE19 cell line (Dunn et al., 1996) was purchased from the American Type Culture Collection (Manassas, VA). Cell culture reagents, fetal bovine serum and chemicals were purchased from Invitrogen–Gibco (Rockville, MD), cell culture plates and filter inserts with 0.4 μm pore size were obtained from Falcon Becton Dickinson (Franklin Lakes, NJ). ARPE19 and RGC5 cells were maintained in Dulbecco's modified Eagle's medium containing 3 mM L-glutamine, 10% fetal bovine serum, 100 U/ml penicillin G and 100 μg/ml streptomycin sulphate at 37 °C in an environment containing 95% O_2 and 5% CO_2. For growth inhibition assays in non-confluent cultures, cell suspensions (5×10^3 cells/ml) were seeded onto 24-well tissue culture plates. After overnight incubation, the cells were washed with phosphate buffered saline (PBS) and exposed to purified TA-containing medium under various conditions.

2.2. Preparation of triamcinolone acetonide

Triamcinolone acetonide (Volon-A 40, Bristol-Myers-Squibb, Munich, Germany) containing 40 mg crystalline suspension was purified according to a standard protocol for human use (Hernaez-Ortega and Soto-Pedre, 2004) and resuspended with culture medium to a stock concentration of 6.25 mg/ml. Final TA concentrations were determined spectrophotometrically at 239 nm (EAR400ATX, Labindustries, Berkeley, CA). The stock solution was vortexed and serially diluted with culture medium up to 2×10^{-7} mg/ml (5×10^{-4} μM).

2.3. MTT cell viability assay

To determine the biological effect of direct TA apposition, the cells were incubated either with or without direct contact to crystalline particles. This was achieved by using a culture plate membrane filter system based on the principle of a Boyden chamber as described previously (Szurman et al., 2006). TA was added either directly on top of the cell layer (adherent epicellular deposits) or on top of the protective filter insert (non-adherent TA) that prevented direct cell contact (Fig. 1).

After 1, 3, 5 and 7 days of incubation with TA at the appropriate concentrations, the cellular activity of mitochondrial dehydrogenase was measured based on the cleavage of the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, #M5655; Sigma, Deisenhofen, Germany). Briefly, the cells were washed with PBS, and then medium-diluted MTT at 0.5 mg/ml was added and incubated for 2 h. After formazan extraction cell proliferation and viability were measured colorimetrically with an ELISA reader (SLT Spectra 400 ATX, Salzburg, Austria) at 570 nm with the correction of interference at 690 nm.

Log–linear TA concentrations were plotted against the relative absorbance reading to calculate the mean inhibitory dose (ID_{50}) for cells exposed to TA with or without protecting filters.

2.4. Recovery assay

The recovery assay was designed to assess the effect of continuous or transient epicellular deposits on retinal cell viability. ARPE19 cells were exposed to TA (0.01–1 mg/ml) either with the drug separated from the cells by a filter or