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Review

On the retinal toxicity of intraocular glucocorticoids

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ABSTRACT

Corticosteroids are hormones involved in many physiological responses such as stress, immune modulation, protein catabolism and water homeostasis. The subfamily of glucocorticoids is used systemically in the treatment of inflammatory diseases or allergic reactions. In the eye, glucocorticoides are used to treat macular edema, inflammation and neovascularization. The most commonly used glucocorticoid is triamcinolone acetonide (TA). The pharmaceutical formulation of TA is not adapted for intravitreal administration but has been selected by ophthalmologists because its very low intraocular solubility provides sustained effect. Visual benefits of intraocular TA do not clearly correlate with morpho-anatomical improvements, suggesting potential toxicity. We therefore studied, non-common, but deleterious effects of glucocorticoids on the retina. We found that the intravitreal administration of TA is beneficial in the treatment of neovascularization because it triggers cell death of endothelial cells of neovessels by a caspase-independent mechanism. However, this treatment is toxic for the retina because it induces a non-apoptotic, caspase-independent cell death related to paraptosis, mostly in the retinal pigmented epithelium cells and the Müller cells.

Contents

1. Corticosteroids

Corticosteroids are hormones produced in the adrenal cortex. They are involved in many physiological responses such as stress, immune modulation, protein catabolism and water homeostasis. This hormone family comprises two subfamilies: mineralocorticoids, involved mostly in electrolyte control and glucocorticoids controlling fat, protein and carbohydrate metabolism.

Therapeutically, glucocorticoids are used systemically in the treatment of inflammatory diseases like arthritis, asthma and allergic reactions. They are also administered topically to the skin, nose or lungs [1].

2. The use of glucocorticoids in the eye

The retina is a neural tissue lining in the inner surface of the eye. In humans and other vertebrates the retina is composed of three

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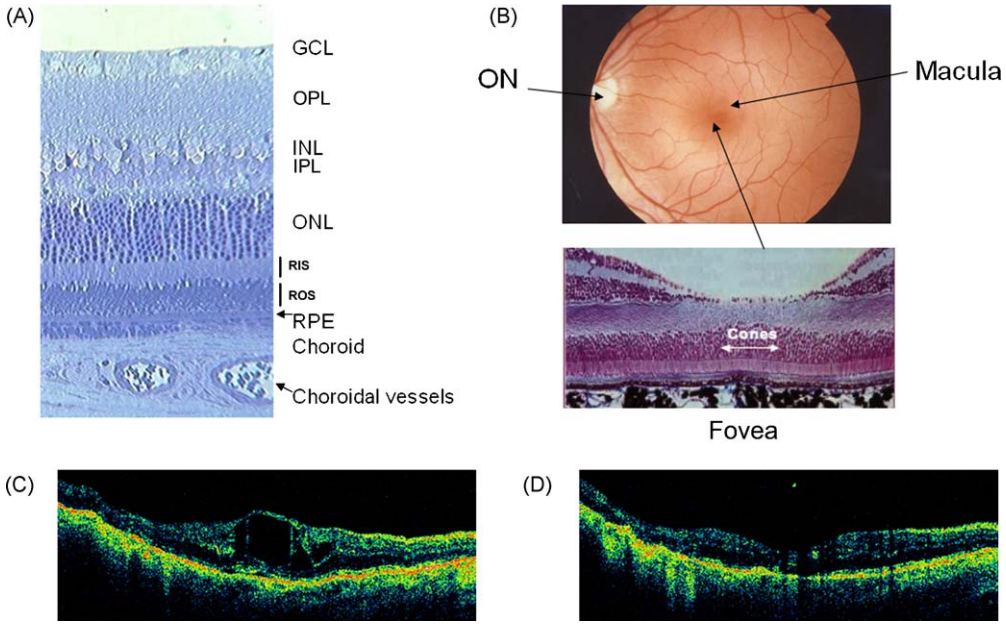

Fig. 1. Anatomy of the retina and macular edema. (A) Cross-section of a rat retina stained with Toluidine Blue. The different cellular layers of the retina are shown: GCL: ganglion cell layer, INL: inner nuclear layer, which contains the bodies of bipolar cells, Müller cells and intermediate cells. ONL: outer nuclear layer, containing the photoreceptor cell bodies, RPE: retinal pigmented epithelium cells. RIS: rod inner segments, ROS: rod outer segments. (B) Fovea and macula. The upper panel shows an eye fundus. On the left there is the optic nerve (ON) and the normal retinal vascularization. In the darker, avascular, area can be seen: the macula. In the center of this area a depression is found: the fovea, the region of highest visual acuity. The lower panel shows a cross-section of the fovea. The different cell layers are pushed to the side and the photoreceptors are directly exposed to light. (C) Optical coherence tomography (OCT) scan of a diabetic patient before triamcinolone acetonide (TA) injection and in (D), 1 week after TA administration.Printed with permission from Miyamoto et al. Ophthalmology 2006;113(November (11)):2048–53. Elsevier Copyright 2006.

layers of nerve cell bodies and two layers of synapses (Fig. 1A). The outer nuclear layer contains the photoreceptors, which are the main light sensitive cells of the retina. Two types of photoreceptors are found: the rods and the cones. Rods provide black-and-white vision, while cones are responsible for the perception of colour. The inner nuclear layer contains bipolar, horizontal and amacrine cells, while the ganglion cell layer contains essentially the ganglion cells. These neural layers are separated by two layers of synaptic contacts, the so-called outer and inner plexiform layers (OPL and IPL respectively) (Fig. 1A). The neural impulse, originating in the photoreceptors, undergoes complex processing by the neurons of the inner nuclear layer and exits the retina though the ganglion cells whose axons form the optic nerve. In the eyes of humans and other primates, the retina has an area where cones are most concentrated, the macula lutea. The macula represents 2–3% of the surface of the retina but it is responsible for 90% of the information coming out of the retina. A small depression is seen in the center of the macula, known as fovea, where only cones are found. This is the area of maximal visual acuity (Fig. 1B).

In order to avoid diffraction of photons by blood cells, the fovea does not contain blood vessels. In addition, the homeostasis of fluids in this region is critical for its proper function. These characteristics are altered in several retinal diseases. For example, an increase in water content in the macula, called macular edema, is one of the leading causes of blindness in diabetic retinopathy. The unbalance between fluid entry and exit in the retina results in increased retinal thickness, intraretinal cysts and subretinal fluid, forming a cystoid macular edema (Fig. 1C). Moreover, a person with macular edema is likely to have blurred vision, leading to social blindness (no recognition of faces, inability to read). In the proliferative forms of diabetic retinopathy (less common since laser photocoagulation has become widely used in industrialized countries), new blood vessels form at the surface of the retina. These new vessels can bleed and induce tractional retinal detachments, ultimately causing total blindness without active treatment.

Intravitreal administration of glucocorticoids is a very commonly practiced treatment for macular edema and less commonly for some forms of neovascularization [2]. The most frequently used corticoid is triamcinolone acetonide (TA). This specific corticosteroid formulation, not adapted and not originally indicated for the eye, has been selected by ophthalmologists because of its sustained effect, due to its very low intraocular solubility. The effect of the intravitreal injection of TA is impressive in some aspects. For instance, a single administration results in a dramatic decrease in macular edema. Fig. 1C and D shows OCT images of the same eye before and 1 week after injection. This effect begins to be noticed already 1 h after the treatment. When used for the treatment of choroidal neovascularizations associated with Age-related Macular Degeneration (AMD), for example, TA decreases the vascular leakage in the short-term. The vascular effects of TA have been confirmed in animal models of choroidal and retinal neovascularization [3].

In spite of these positive effects of TA on retinal anatomy, the improvement of long-term visual acuity is observed in only 50% of patients treated for macular edema [4]. When associated with other anti-vascular drugs for the treatment of neovascular AMD, severe side effects have been reported [5].

In addition, the safety of TA is still a subject of controversies. In particular, it is not clear whether the observed toxicity results from the glucocorticoids themselves (including TA) or is a consequence of the use of specific vehicles. This led us to investigate the toxicity of glucocorticoids in the retina, with a particular emphasis on TA.

3. The toxicity of TA in the retina

Previous research has allowed the toxicity of TA in non-ocular tissues to be minimized. However, the toxic effect of TA is a fact and

Table 1
Retinal toxicity of TA in vivo.

	Experimental settings	Injection	Length	Analysis	Results and conclusions
McCuen et al. [51]	IVT rabbit Eye fundus Intraocular pressure Histology	TA1 mg/NaCl	3 months	Slit lamp optical and electronic microscopy ERG	No toxicity
Hida et al. [40]	IVT rabbit Eye fundus Histology	Vehicles of Kenacort	2 weeks	Slit lamp optical microscopy	Benzyl alcohol toxicity
Kilvilcim et al. [53]	IVT rabbit (after vitrectomy +silicone) Eye fundus Histology	1, 2 or 4 mg TA or Kenakort	140 days	Slit lamp optical and electronic microscopy ERG	No toxicity
Dierks et al. [54]	IVT rabbit Eye fundus Histology	4 mg whole Kenacort or w/o vehicles Vehicles alone	7 days	Slit lamp optical microscopy ERG	No toxicity
Kai et al. [55]	IVT rabbit Intraocular pressure Histology	4 or 25 mg whole Kenacort or w/o vehicle	2 months	Optical and electronic microscopy ERG	Increase in intraocular pressure Decrease of ERG Toxicity of vehicle but not TA
deKozak et al. [56]	Subretinal injection Rabbit Histology	2 mg Kenacort w/o vehicle	3 months	Optical and electronic microscopy ERG OCT	Toxic for the RPE and outer rod segments of photoreceptors Crystals of TA toxic for the retina
YU et al. [57]	IVT rabbit Histology	0,5, 1, 4, 8 and 20 mg whole Kenacort or vehicles alone	14 days	Optical and electronic microscopy ERG	Toxic for the RPE and outer rod segments of photoreceptors above 4 mg vehicles not toxics
Morrison et al. [58]	IVT rabbit Histology	0.022,0.073, 0.222 and 0.733% in benzyl alcohol	14 days	Optical and electronic microscopy ERG	Toxic for the RPE and outer rod segments of photoreceptors above 0.073
Lang et al. [59]	IVT rabbit Eye fundus Histology	4 mg whole Kenacort or vehicles alone	6 weeks	Optical microscopy ERG	Retinal toxicity due to vehicles
Albini et al. [60]	IVT rabbit	4 mg ken complete	17 weeks	Optical and electronic microscopy Histology ERG	No toxicity
Ruiz-Moreno et al. [61]	IVT rabbit Histology Immunohistochemistry	4, 20 whole Kenacort	28 weeks	Optical and electronic microscopy ERG	No toxicity
Macky et al. [62]	IVT/ceil Lapin Eye fundus Histology	0.1 ml vehicles of Kenacort	6 weeks	Optical and electronic microscopy ERG	Toxic for all the layers of the retina
Ruiz-Moreno et al. [63]	IVT rabbit Histology	4, 20 or 30 mg TA Benzyl alcohol	28 days	Optical and electronic microscopy ERG Immunohistochemistry	No toxicity
Yi et al. [64]	IVT horse Eye fundus Histology	10, 20 or 40 mg TA	7 or 21 days	Biomicroscope ERG Slit lamp	No toxicity

IVT = intravitreal administration, ERG = electroretinogram, OCT = optical coherence tomography.

has been initially reported after administration to chick embryo where it is responsible for lymphocyte toxicity and malformations [6]. Several authors have also performed studies on TA toxicity in the retina. A non-exhaustive list is shown in Table 1. Overall, this table indicates that the results are inconclusive, spanning from toxic to non-toxic effects for TA, or pointing to the toxic effects of preservative used in the pharmaceutical preparations. One of the most limiting points of these studies is that no mechanism of cell death could be identified, thus questioning the suspected toxicity of TA.

A similar situation can be seen concerning the control of neovascularization with this drug. TA has been shown to down regulate the expression of tumour necrosis factor α, vascular endothelial growth factor (VEGF), interleukin 1, and matrix metalloproteinases, all of them influencing neovascularization [7,8]. TA has also been reported to have an anti-proliferative effect on endothelial cells [9,10]. So far, the antiangiogenic/angiostatic activity of corticosteroids results from a combination of factors. It remains, however, unknown if TA can directly induce vascular endothelial cell death.

3.1. The mechanisms of cell death

During most pathological processes cell death is essentially carried out through either passive or active mechanisms. Passive mechanisms are involved in extreme cell injury and end up into the release of the entire cell contents in the tissue. This process of cell death is called necrosis. By contrast, the cells suffering from mild or light injury die through an active and programmed cell death [11] that will protect the neighbouring cells from extensive damage. Caspase-dependent apoptosis is the most widely studied and characterized pathway of programmed cell death (for a review see [12]). However, other forms of active cell death are now recognized and their role in tissue homeostasis and pathology is being unveiled [13–15]. Thus, other apoptotic caspase-independent mechanisms (also called apoptosis-like mechanisms) have been

described. Caspase-independent cell death mechanisms result from the activation of other proteases such as calpains [16] cathepsins [17,18] or serine proteases [19,20] that in turn activate other effectors of cell death such as apoptosis-inducing factor (AIF) [21] or L-DNase II [22,23]. In recent years other processes have been considered as involved in mechanisms of cell death. Autophagy, for instance, is an evolutionarily conserved mechanism allowing the elimination of unnecessary or damaged organelles and the recycling of their proteins. In neural cells, autophagy is essential for cell survival, but when over activated may lead to cell death [24,25]. Recently, an additional cell death program, initiated in the cytoplasm and named paraptosis, has been characterized [13]. This type of cell death has been first described after the activation of the insulin-like growth factor receptor [26–28]. A similar death phenotype is initiated after the activation of neurokinin-1 receptor by its ligand, substance-P [29]. These types of cell death are insensitive to broad-range caspase inhibitors and present an extensive cytoplasmic vacuolization [29,30]. Paraptosis also involves the activation of MAP kinases and can specifically be blocked by AIP-1/Alix [13]. This last characteristic is, up to now, the only functional evidence that allows this type of cell death to be identified.

3.2. TA induces cell death in endothelial cells and neovessels

We have recently shown that TA strongly affects endothelial cell viability when assayed on bovine retinal endothelial cells (BREC) [31]. In these experiments, the percentage of living cells was reduced to almost 50% after exposure to TA for 24 h at a concentration of 1 mg/ml, which is the concentration normally used in human clinics. The rate of survival was further reduced as exposure time increased. This effect was similar regardless of whether confluent or non-confluent cells were used, suggesting that proliferation is not a major process controlling the size of the studied cell population. However, this does not mean that proliferation is not affected. Indeed, the evaluation of the rate of DNA synthesis using BrdU incorporation revealed a reduced proliferation [31]. However, the effects on proliferation could not explain the magnitude of cell loss quantitatively. To test the hypothesis that cell death is contributing to the observed, TA-induced cell loss, we first measured the amount of free lactate dehydrogenase (LDH) in the culture medium. This enzyme was used as a marker of passive cell death by necrosis. We found that necrosis-related LDH release was increased. However, a decrease in the size of the cell population through the combined decrease of proliferation and increased necrosis could still not entirely explain the magnitude of the observed cell loss. Taken together, this evidence suggests that additional mechanism of cell death, via active pathways, is also involved. Indeed, our experiments showed that these cells expose phosphatidyl serine on the outer side of the plasma membrane, as revealed by annexin V binding. However, neither activation of the central caspase of the classic apoptotic pathway, caspase 3, nor TUNEL labelling (indicating a degradation of DNA in the nucleus) was seen. These results suggest that the classic apoptotic pathway of cell death is not activated.

The presence of cell death in TUNEL negative cells may be explained by either a lack of DNA degradation or by a degradation of DNA which cannot be labelled with this technique. This happens for instance when L-DNase II, an enzyme activated by serine proteases, produces 3′P ends which cannot be labelled by the terminal transferase, enzyme used in TUNEL assays. It is worth noting that terminal transferase can only add nucleotides to 3′OH ends. Consistently, L-DNase II, an acid endonuclease, cleaves DNA and releases 3′P ends that cannot be labelled by the TUNEL technique. In our experiments, LEI/L-DNase II was associated with apoptosis of BRECs after 72 h of TA exposure (Fig. 2). This explains why these cells, displaying apoptotic morphology and annexin V staining, but yet remain TUNEL negative. In addition, as TUNEL is the common technique used to assay the induction of cell death in a tissue, many dying cells remain unrevealed. In addition to LEI/L-DNase II-induced cell death where L-DNase II is translocated to the nucleus upon its activation by serine proteases, AIF is also targeted to the nucleus (Fig. 2). Interestingly, it has to be stressed that AIF is translocated to the nucleus only after L-DNase II. Indeed, double immuno-labelling of these two effectors of apoptosis showed that cells can display activated L-DNase II without nuclear translocation of AIF. The opposite was never seen, suggesting that AIF is involved in this cell death only at later stages.

According to these data, activation of a caspase-independent apoptosis-like cell death is responsible for the death of endothelial cells. Previous studies performed in our laboratory showed a

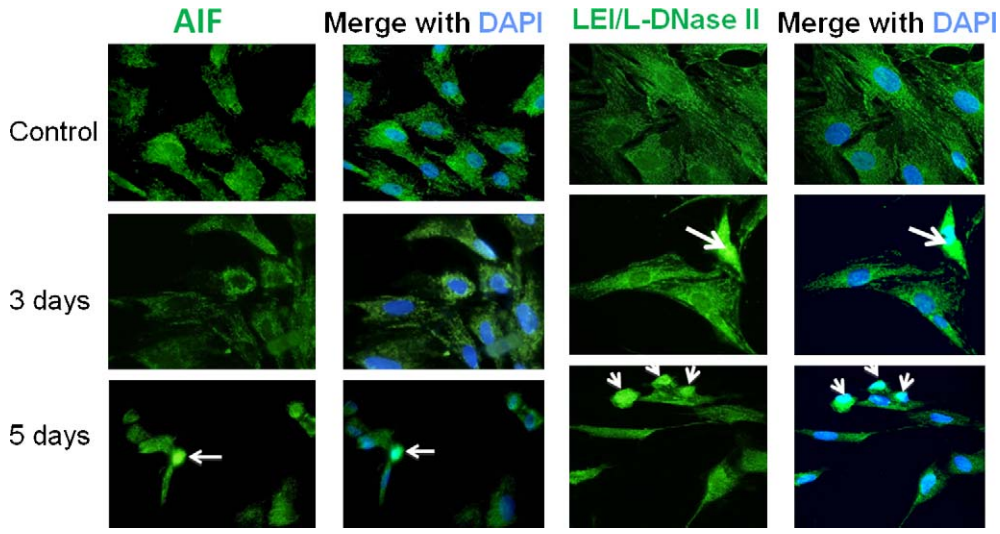

Fig. 2. AIF and L-DNase II activation in TA-treated BRECs. (Right panel) Apoptosis-inducing factor (AIF) immuno-labelling was performed in control cells treated with the TA vehicle (1% ethanol), where cytoplasmic labelling can be seen. This is also the case in cells treated with 0.1 mg/ml TA for 3 days. After 5 days of TA treatment, cells with condensed nuclei (arrows) show nuclear staining for AIF. Nuclei were identified by DAPI staining. (Left panel) L-DNase II activation in TA-treated BRECs. LEI/L-DNase II immuno-labelling was performed in control cells as before and cytoplasmic labelling is seen. Cells treated with 0.1 mg/ml TA for 3 or 5 days have condensed nuclei (arrows) and present nuclear staining of LEI/L-DNase II, indicating L-DNase II activation.Reproduced with permission from Mol Vis 15;2009:2634–48.

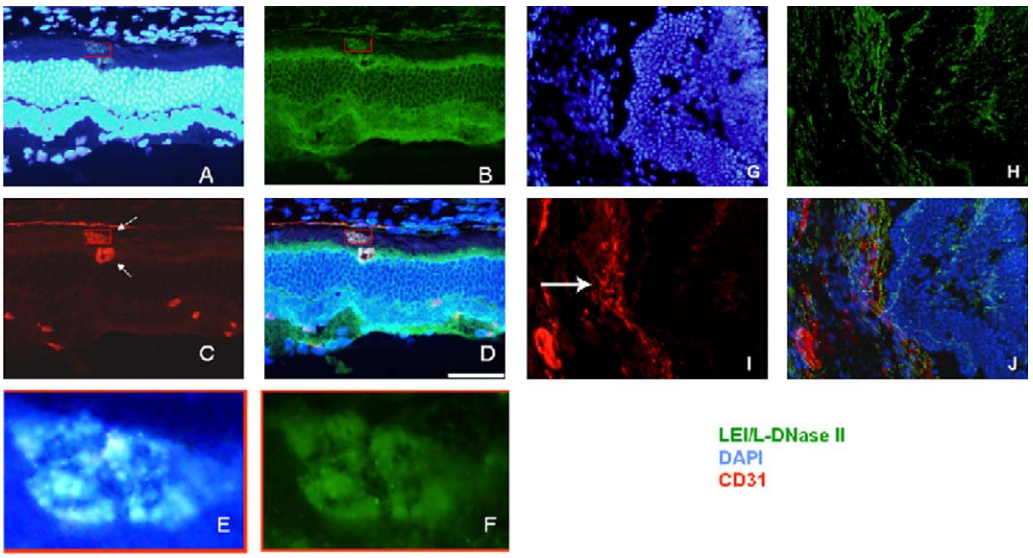

Fig. 3. TA effects on neovessels in vivo: 10- to 12-week-old C57/bk6 mice were treated with an argon laser of 50 μ, 400 mW, 0.05 s (burning rate 0.40, 2 weeks after burning), 15 μg of TA was injected in the eye (A–F) or a saline solution was used for control animals (G–J). Mice were sacrificed using CO_2 1 month later. The eyes were cryosectioned and immunostained with DAPI (A and G) to identify nuclei, anti-LEI/L-DNase II (B and H) and anti CD31 (C and I), which identifies endothelial cells. (D) and (J) depict the merged images. Neovessels are present in the outer nuclear layer, a zone that is normally avascular (white arrows on (C) and (I)). (E) and (F) are higher magnifications of the red square areas. The chromatin in the endothelial cells is disrupted and a clear nuclear labelling of L-DNase II is seen. Scale bar represents 50 μm.

decrease in neovascularization after treatment with TA in an induced model of choroidal neovascularization [32]. To induce the growth of neovessels C57/B6 mice were exposed to an Argon laser at 400 mW, a procedure that burns the retina and destroys the pigmented epithelium in a reduced area (50 μm). Two weeks after this treatment, the eyes were injected intravitreally with TA. The effects were evaluated 1 month later. Fig. 3 shows a burned retinal area where, the vessels are labelled with CD31. The neovessels that are growing in a normally avascular area are shown by an arrow. It can be seen that after TA treatment the nuclei of the endothelial cells of these vessels are fragmented and labelled with anti-L-DNase II, a feature not seen in control eyes burned but not treated with TA. This suggests that in vivo TA has the same effect on endothelial cells as in vitro.

Taken together these data indicate that TA relieves neovascularization by decreasing proliferation and inducing caspase-independent cell death of endothelial cells. This represents a potentially beneficial effect in the treatment of neovascularization. The question which remains to be answered is then: is TA safe for the retina?

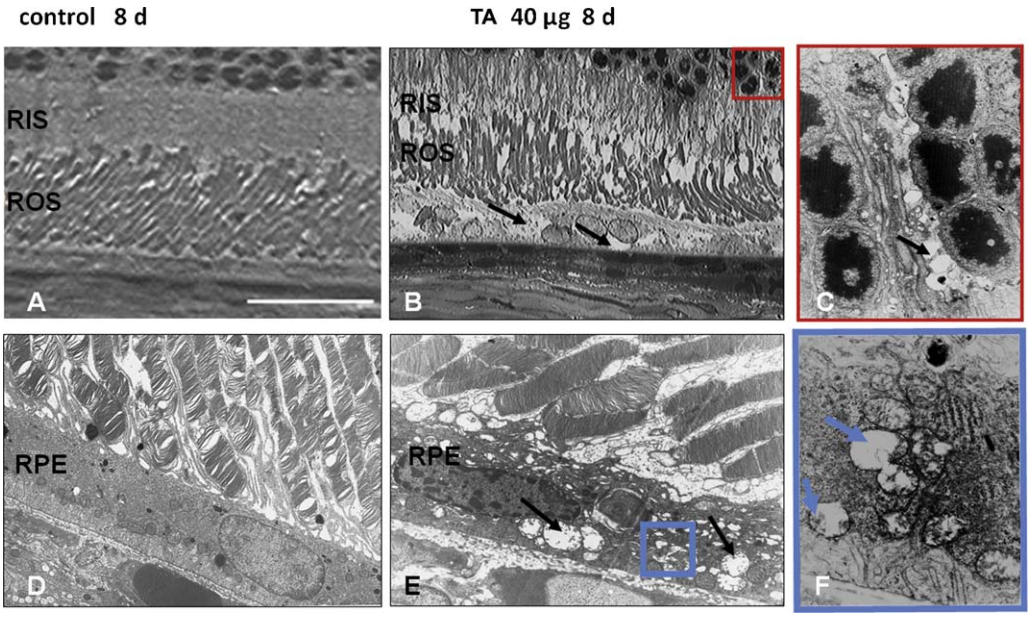

Fig. 4. Structural alterations of the rat retina 8 days after intravitreous injection of TA. (A) Retina from a PBS-treated rat (control). (B) Retina from TA-injected eyes. Black arrows show vacuoles and enlarged retinal pigment epithelium (RPE) cells. Scale bar represents 10 μm. Lower panels are ultrathin sections. (D) Retina from a PBS-treated rat (control). (E) Retina from TA-injected eyes. (C) and (F) are higher magnifications of degraded mitochondria (F, arrows). (C) Vacuoles (arrows) are also observed in retina glial Müller cells prolongations. Note preservation of photoreceptor nuclei. ROS indicates rod outer segments, RIS indicates inner segments. RPE indicates retinal pigmented epithelial cells.Reproduced with permission from Ref. [33].

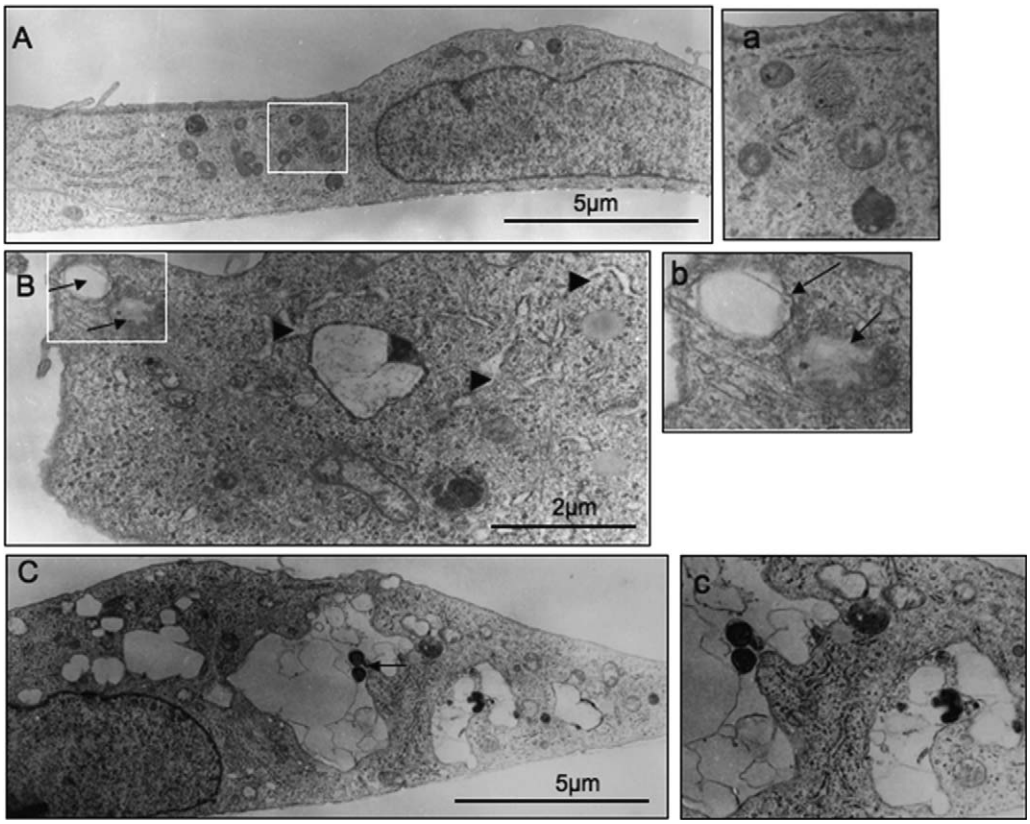

Fig. 5. Transmission electron microscopy observations of TA-treated ARPE-19 cells. (A) Control ARPE-19 cells. (B and C) ARPE-19 cells TA-treated with 0.1 mg/ml (B) and 1 mg/ml TA (C), showing membrane limited (arrows) or unlimited vesicles and dilated endoplasmic reticulum (arrowhead). In (C), a more advanced stage of degeneration is represented.Reproduced with permission from Ref. [33].

3.3. The retinal toxicity of TA

In order to investigate the effects of TA on the rat retina, we injected TA into the vitreous of rat eyes [33]. While the control eye, injected with a saline solution, showed no abnormalities (Fig. 4), the eyes receiving TA showed marked morphological changes 8 days after injection mostly in RPE (retinal pigmented epithelial cells) cells (Fig. 4). The photoreceptors outer segments were disorganized but the cell bodies were preserved. The RPE layer was not interrupted but the individual cells were enlarged and presented cytoplasmic vacuoles and increased microvilli, a morphology suggesting a replacement of lost RPE cells by enlargement of the neighbouring cells. These modifications were confirmed by electron microscopy (Fig. 4). The cells of the RPE were not the only cells modified. Vacuolization within the cytoplasm was also observed in Müller cells. In these cells, the vacuoles were essentially seen in their prolongations at the outer nuclear layer level. These morphological changes suggested a cellular stress but they were not in accordance with an apoptotic cell death. Moreover, immunostaining of cryosections of these retinas with anti-activated caspase 3 and TUNEL assays were negative. No nuclear translocation of L-DNase II or AIF was found suggesting the absence of their activation [33]. In order to explore the effects of TA on these cells, we investigated its actions in vitro in cultured RPE and Müller cells. It is interesting to note that the morphological effects observed in vitro were similar to those seen in vivo. Fig. 5 shows the electronic micrography of a cultured RPE cell where the presence of vacuoles is also induced by TA. In cultured cells TA induced a dose-dependent reduction in cell viability of both cell types with the loss of about 50% of living cells at 0.1 mg/ml concentration, indicating that TA was highly toxic for these cells. However, as observed with retinal sections, no TUNEL positive cells could be detected (Fig. 6). In addition, annexin V did not bind to cells exposed to TA. Other biochemical markers of apoptosis, such as activation of caspase-3 and release of cytochrome *C* from the mitochondria, were also negative. These data excluded caspase-dependent apoptosis as a potential mechanism for the observed cell death induced by TA. Two markers of caspase-independent apoptosis (L-DNase II and AIF) were further investigated and their activation was also not detected (Fig. 6). The morphological modifications observed in these cells are indicative of different types of cell death: necrosis, autophagy and paraptosis. To investigate the possible involvement of a passive cell death, i.e. necrosis, we measured the LDH cell release. Only cells exposed to very high concentrations of TA, inducing the formation of crystals, triggered necrosis. In the absence of crystals, this type of cell death was not induced.

Autophagic activity was investigated by using both monodansylcadaverine (a compound staining acidic vesicles) and anti-LC3, an antibody staining mature autophagic vacuoles [33]. Only some of the vacuoles were stained with monodansylcadaverine in TA-treated RPE cells. Most importantly, MAP-LC3 labelling was not significantly different in TA-treated and control cells, indicating that autophagy is not activated. We finally decided to explore the possible involvement of paraptosis.

Several cues were in favour of the activation of paraptosis in TA-treated cells: the observed cell death occurs with no DNA condensation, no formation of apoptotic bodies, no caspases activity and no DNA degradation. However, extensive cytoplasmic vacuolization is seen. In order to test this hypothesis, we over-expressed AIP-1/Alix in these cells. As shown in Fig. 7, over-expression of AIP-1/Alix in wild type significantly reduced TA-induced toxicity. This protection is lost if only the C-terminal part of the protein is expressed.

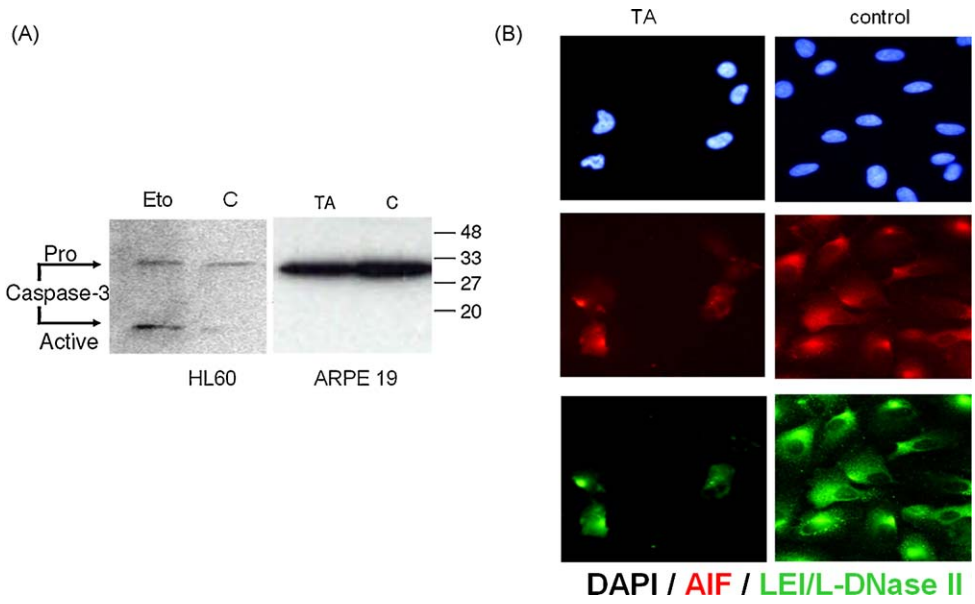

Fig. 6. The cell death activated in ARPE-19 is caspase-independent. (A) Subconfluent ARPE-19 cells were incubated for 72 h in the presence 0.1 mg/ml triamcinolone. Protein extracts were separated in 4–10% PAGE, transferred to nitrocellulose and revealed with anti-caspase 3. No activation of caspase 3 is seen in TA-treated cells. Apoptosis induced in HL-60 with etoposide (50 μM for 24 h) was used as positive control. (B) Triamcinolone-treated or control ARPE-19 cells were stained with DAPI or immunostained with anti-AIF (red) or anti-LEI/L-DNase II (green). No nuclear translocation of these apoptosis markers was seen, neither in cells with normal morphology, nor in cells with an altered nuclear morphology.Reproduced with permission from Ref. [33].

Taken together these data indicate that when exposed to TA, RPE and RMG cells die by paraptosis, a non-classical form of cell death.

4. The use of glucocorticosteroids in the eye revisited

Contradictory observations and discrepancies regarding the clinical safety of intravitreous TA toxicity have been published [34–36], depending mostly on the techniques used to detect toxicity. In the rat eye, using electron microscopy we could detect clear lesions in RPE and in Müller cells 8 days after injection of TA. However, all currently used immunohistochemical markers for apoptosis or autophagy (activated caspase-3, TUNEL assay, and MAP-LCA) are negative. This may explain why other authors previously failed to clearly detect any cell death even when toxicity markers have been observed. Previous in vitro experiments have also shown that direct incubation of cells with crystalline TA induces activation of caspases and stress proteins [37,38]. This effect was lost when the crystals were separated from the cells by a microporous membrane [39]. However, in these experiments, other potential mechanisms of cell death were not explored. It is interesting to note that this situation does not seem to be exclusive

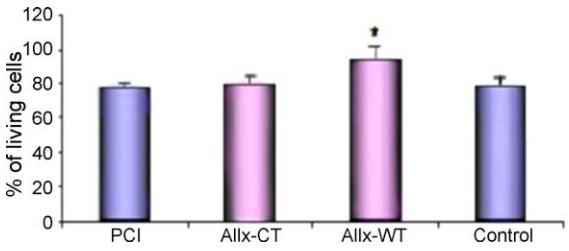

Fig. 7. Paraptosis in TA-treated ARPE-19 cells. Effect of Alix overexpression on TA induced cell death. ARPE-19 cells were nucleofected with Alix-WT or with its C-terminal moiety. Controls were run with untransfected cells or in cells transfected with the PCI empty vector. Forty-eight hours after transfection cells were treated with TA and cell survival was measured with the 3-(4,5-dimethylthiazol-2-yl)-2,5-phenyltetrazolium bromide test method. Alix-CT has no effect on cell survival but Alix-WT protects cells form death. * indicates a $p < 0.05$.Reproduced with permission from Ref. [33].

for the retina, but might also explain the "absence" of cell death in various brain regions reported in some neurodegenerative pathologies [41,42] where, as in the retina, no classical apoptosis markers could be detected. The involvement of non-classical cell death effectors, similar to that discussed in this report, should be performed in the relevant paradigms of neurodegenerative pathologies occurring in the absence of the classical apoptosis.

The cell death induced by TA is related to paraptosis. It is worth noting that paraptosis takes place during cell differentiation in the development of the nervous system as well as in some cases of neurodegeneration [43]. This indicates that the investigation of different cell death mechanisms is critically important in evaluating the toxicity of a product or even its therapeutic efficacy. This is clearly the case for the anti-vascular activity of steroids.

Glucocorticoids were known from a long time to induce vascular effects. Actually, the McKenzie test, based on skin bleaching [44,45], has been used to establish the classification of glucocorticoid potency and cutaneous absorption. This test uses the visual quantification of the skin whitening after 8–48 h of exposure to different glucocorticoids applied topically under occlusion. Although this test is widely used, the exact mechanism of skin whitening remains unknown. The effects of steroids on growing vessels have been described in a seminal paper by Folkman and Ingber [46]. These authors showed that dexamethasone or hydrocortisone induces the regression of neovessels in the chorioallantoid membrane. They have demonstrated that the vascular action of these drugs is related to their effect on the basal membrane of growing vessels. Further studies have shown that steroids inhibit the proliferation of human endothelial cells [47]. In our work we showed a decrease of proliferation but also an induction of cell death. Here again, classical mechanisms of cell death, caspases-dependent apoptosis or necrosis, are not activated and the use of the classical TUNEL technique cannot accurately reveal dying cells. Moreover, TA has also been shown to induce vascular changes, at the level of the choroids [48–50] in normal eyes. The potential toxic effect of TA on resting versus proliferating choroidal neovessels is currently under investigation.

Glucocorticosteroids are frequently used in a systemic way and injected intravenously. Our results raise the question of the effect

of these bolus injections on vasculature. Endothelial cell damage has been reported in veins of rabbits treated with methylprednisolone. This observation is in agreement with the superficial skin ecchymoses frequently seen after long-term corticotherapy [52]. Also the question of the effects of corticosteroids on other endothelia of the microcirculation has to be addressed. In our experiments, we found a relatively selective effect of TA on the choriocapillaries as compared to the retinal vasculature. In fact, these two types of capillaries are quite different. Choriocapillary endothelium is fenestrated and much less protected than retinal capillaries, which are surrounded by pericytes, astrocytes, and retinal Müller glial cells. Moreover, TA could also exert an indirect effect through the downregulation of COX2 in RPE cells [31]. This evidence points to the fact that the results obtained on one type of these two capillaries cannot obviously be extrapolated to the other one. The deleterious effects of chronic use of glucocorticoids have been described in other systems. For instance, the long-term use of topical steroids on the skin is often accompanied by severe and often irreversible effects as skin atrophy [65]. This is also the case for the muscle, where the use of glucocorticoids is a common cause of muscle atrophy [66]. Whether these tissues are also suffering cell death by non-canonical mechanisms is not known. This issue is critical in the evaluation of the safe use of theses valuables drugs and will be matter of a future investigation.

5. Conclusion

Our work shows for the first time that glucocorticoids limit neovascularization by inducing cell death in endothelial cells. We also demonstrate a direct retinal toxicity of glucocorticoids. In both cases, the activated cell death mechanism is caspase-independent, therefore undetectable when using techniques that detect this form of cell death. Taking into account that caspase-independent cell death may occur without any macroscopic reaction, in the long-term, such cell death may remain undetectable in the routine clinical practice and promote deleterious consequences on visual acuity.

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