Title: Glucocorticoids exert direct toxicity on microvasculature: analysis of cell death mechanisms.
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Journal: Toxicological sciences : an official journal of the Society of Toxicology
Year: 2015 Feb
Volume: 143
Issue: 2
Pages: 441-53
DOI: 10.1093/toxsci/kfu243

Glucocorticoids exert direct toxicity on microvasculature: Analysis of cell death mechanisms.

Running title: Glucocorticoids toxicity on vascular endothelium.

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Abstract:

Glucocorticosteroids (GCs) are routinely administered systemically or injected into the eye when treating numerous ocular diseases, however their toxicity on the retinal microvasculature has not been previously investigated. In this paper, the effects of Hydrocortisone (Hydro), Dexamethasone, Dexamethasone-Phosphate and Triamcinolone Acetonide (TA) were evaluated on the microcirculation of human skin, bovine retinal cells and, *ex*-vivo, on flat mounted rat retinas.

The degree of GCs induced endothelial cell death varied according to the endothelial cell type and GCs chemical properties. GCs toxicity was higher in skin microvascular endothelial cells and for hydrophobic GC formulations. The mechanism of cell death differed between GCs, Hydro and TA activated the Leukocyte Elastase Inhibitor/L-DNase II pathways but did not activate caspases.The mechanisms of cell death observed in cell cultures were similar to those observed in rat retinal explants. Taken together these results indicate that a particular attention should be paid to the potential vascular side effects when administrating GCs clinically and in particular when developing sustained-release intraocular devices.

Keywords: Glucocorticoids, endothelial cell, retinal microvasculature, apoptosis, dermic endothelial cell.

Introduction

Glucocorticoids hormones easily cross cell membranes due to their lipophilicity and exert physiological effects in almost every tissue of the human body (Cockrem 2013, Walker, *et al.* 2012). Synthetic or semi-synthetic glucocorticoids (GCs) aim to optimize the immunosuppressive, angiostatic and anti-inflammatory properties of cortisol, whilst reducing their mineralocorticoid side effects. GCs remain the first-line therapy in number of inflammatory conditions and are administered locally or systemically (Barnes 2005, Logie, *et al.* 2010). Recognized side effects of GCs include microvascular fragility, atrophy as well as reduced microvascular density in skin, bone and muscle (Anacker, *et al.* 2011, Schacke, *et al.* 2002, Torriglia, *et al.* 2010).

Folkman et al. demonstrated that Hydrocortisone (Hydro) and Dexamethasone (Dexa), at µg/µl concentrations, prevent heparin-stimulated angiogenesis in the chick chorioallantoic membrane and induce the regression of capillaries within the surrounding tissue (Folkman and Ingber 1987, Folkman, *et al.* 1983). Later, Small et al. showed that corticosterone (300–600nM) inhibits angiogenesis *in vitro* and *in vivo*. In addition, inhibition of 11beta-hydroxysteroid dehydrogenase type 1 favors corticosterone production and enhances angiogenesis providing evidence for the angiostatic role of endogenous corticosteroids (Small, *et al.* 2005). More recently, the mechanisms of the angiostatic effects of physiological doses of the endogenous corticosterone have been studied in more details, revealing a receptor-mediated effect on Vascular Endothelial Growth Factor production, activation of Matrix Metallo peptidase 2 and the inhibition of endothelial cells tube-like structure formation (Shikatani, *et al.* 2012). Systemic administration of GCs triggered cell death of macrophages (Schmidt *et al.*, 1999), human epithelial cells of the lens (Sharma, *et al.* 2011), pericytes

(Katychev, *et al.* 2003) and induced necrosis of microvessels and osteocytes in the co-femoral articulation and bruises (Allen, *et al.* 2003, Hu, *et al.* 2006).

The possibility that higher doses of corticosteroids may exert further direct effects on an established microvasculature has not been extensively explored.

In ophthalmology, GCs are commonly used not only for the treatment of ocular inflammation but also to manage macular edema of inflammatory and non-inflammatory origins such as diabetic retinopathy or retinal vein occlusion. As chronic use is required, high doses of the hydrophobic Triamcinolone Acetonide (TA) or polymeric implants releasing Dexa for sustained periods are used in order to reduce the frequency of intraocular administrations (Behar-Cohen 2011). In addition, ocular tissues are resistant to the use of GCs due to the high concentration of natural steroid (Knisely, *et al.* 1994), explaining the high concentrations (about 1mg/ml) used clinically. GCs exert rapid and striking effects on retinal edema (Miyamoto, *et al.* 2006) but are associated with potential side effects on the posterior segment of the eye including a reduction of the choroidal vascular network, a decreased retinal vessels diameter in rats and a decrease in the retinal vascular density in the developing retinal vasculature (Hartnett, *et al.* 2006, Valamanesh, *et al.* 2009). Whether GCs can directly affect endothelial cell viability has not been fully explored.

On the one hand, as compared to bone, muscle or skin where vasculature is constantly remodeled, retinal endothelial cells proliferate at a very slow rate in normal conditions (Joussen, *et al.* 2007). On the other hand, GCs failed to demonstrate anti-angiogenic effects in retinal or choroidal neovascularisation (Chan, *et al.* 2011, Geltzer, *et al.* 2013) suggesting that GCs may exert different effects on endothelial cells from different tissue origin. We have previously shown that clinical doses of intraocular TA induced a caspase independent apoptosis associated with the absence of TUNEL labeling on bovine retinal endothelial cells in culture and in retinal neovessels (Torriglia, *et al.* 2010, Valamanesh, *et al.* 2009). This

suggested that non classical mechanism of cell death like autophagy, caspase independent apoptosis or necrosis (Galluzzi, *et al.* 2012) could be involved in this toxicity.

Taken together, all these results suggest that the molecular mechanisms underlying GCs effects on the vasculature are complex and involve different cellular and molecular pathways depending on the endothelial cell type, the GC concentration and duration of exposure.

The aim of this study was to compare the potential toxicity of commonly used GCs (Le Jeunne 2012) on vascular endothelial cells of different origin and to investigate the underlying mechanisms of toxicity for the different GCs. This is a very important point since the evaluation of toxicity relays frequently in caspase-dependent markers and could be underestimated if other molecular mechanisms are involved. The relevance of our *in vitro* findings was assessed by analyzing GCs effects on rat retinal explants.

MATERIALS AND METHODS

Animals

Adult males Lewis rats (6-8 weeks old, Janvier, Le Genest-Saint-Isle, France) were used for the ex-vivo experiments. Rats were sacrificed by carbon dioxide inhalation. All experiments were performed in accordance with the European Communities Council Directive 86/609/EEC and approved by the local ethical committee of University Paris Descartes.

HMEC Culture

All culture media and additives were purchased from Invitrogen (Cergy Pontoise, France). All glucocorticoids (GCs) were purchased from Sigma-Aldrich (Saint-Quentin Fallavier France). Human dermal microvascular endothelial cells (HMEC) is a transformed cell line which has been transfected with a PBR-322 plasmid containing a region encoding for the SV40 (Simian Virus) gene (Ades, *et al.* 1992). Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2. They were cultured in the Endothelial Basal Medium supplemented with L-Glutamine and Hepes 15 mM, 10 % complement-free Fetal Calf serum (DFCS) and 1% streptomycin (100 µg/ml) – penicillin (100 units/ml). Confluent HMEC cells were treated with the LC_{50} of GCs: 275 µM Hydrocortisone (Hydro), 255 µM Dexamethasone (Dexa), 230 µM Triamcinolone Acetonide (TA) and 2 mM Dexamethasone-Phosphate (Dexa-Ph) in media supplemented with 2% of DFCS for 24 h. Although cell survival reaches a plateau with Dexa-Ph, 2 mM was the concentration giving the most reproducible results. The hydrophobic GCs (all except for Dexa-Ph) were previously dissolved in ethanol 100% then in the medium (ethanol final concentration 1%). This step was introduced in order to have a known concentration of the GCs in the media of treatment. Actually, hydrophobic GCs as TA, for instance, precipitate in aqueous medium. This false the real concentration of the GC and generates crystals that precipitate, stick to cells and cause a mechanical damage.

BREC Culture

Bovine Retinal Endothelial cells (BRECs) were isolated from the Bovine retinal microcirculation as previously reported (Capetandes and Gerritsen 1990). BREC were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with GlutaMAX, 4.5 g /L Glucose, 10% decomplemented fetal calf serum (DFCS), 1% streptomycin (100 µg/ml) – (100 Unites/ml) penicillin, 0.5% Fungizone-Amphotericine and 20 ng/ml FGFb. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2. Confluent BRECs cells were treated with LC_{50} GCs 1.37 mM for Hydro, 765 µM Dexa, 460 µM TA and 2 mM for Dexa-Ph in media supplemented with 2% DFCS for 24 h. The hydrophobic GCs (all except for Dexa-Ph) were previously dissolved in ethanol 100% then in the medium (ethanol final concentration 1%). The choice of this cell line was forced by the difficulty of obtaining an equivalent from human origin.

Rat Retinal Explants

After enucleation, rat retinas were isolated immediately under aseptic conditions. They were flattened by 4 orthogonal incisions, then transferred onto a Cyclopore 0.2 µm polycarbonate membrane (Whatman, Maidstone, England), and mounted with the vitreal side up. The support membranes were placed in 6-well tissue culture plates containing 2 ml DMEM supplemented with 10% steroid-free FCS, 1% penicillin-streptomycin and 0.1% Amphotericin-B. Explants were treated with LC_{50} of BREC cells as described for BREC cultured cells.

MTT ASSAY

The MTT assay is a colorimetric test that was developed to determine the survival and growth of eucaryotic cells in proliferation or to evaluate cytotoxicity (Mosmann 1983).The ability to reduce the Tetrazolium salts by HMEC and BREC cells was used to evaluate the toxicity and LC_{50} of GCs. Briefly, cells were seeded in 24-multiwell plates at a density of $5x10^4$cells in

1ml of complete medium. Confluent cells were treated during 24 h with increasing concentrations of CGs. At the end of the treatment, 250 µl of CellTiter 96 Non-Radioactive Cell Proliferation Assay (MTT; Promega, G4000) (5 mg of MTT per ml of phosphate-buffered saline) were added to each well, including six wells that contained only medium or medium containing 1% ethanol (controls). Plates were then incubated for 1 h at 37°C in the dark and analyzed with a microplate reader (BioRad Benchmark, Paris, France) at 570 *vs* 630 nm. Experiments were performed in triplicate and repeated three times.

Caspases inhibition

30 min prior to treatment HMEC cells were incubated in 10µM ZVAD-FMK (Benzoyloxycarbonyl-val-ala-asp-fluoromethyl ketone) (ApexBio A1902). Treatment was initiated by adding GCs at the CL_{50} concentrations in the same medium. Controls included 0.2% DMSO, the vehicle of ZVAD (not shown). Etoposide treatment, 100µM for 24h, was used as a positive control of caspase activation. Cell survival was measured by the MMT method.

Internucleosomal DNA degradation

Internucleosomal DNA degradation assay was performed as already described by Zhua et al (Ning Zhua 1997). Briefly, cells from each condition were obtained by scraping in culture medium. A cell pellet was obtained by centrifugation at 173g, then the pellet was extracted in 30µl of Sarkozyl (50 mM of Tris,10 mM of EDTA, 2% of N-Lauryl sarcosine at pH 7.5) and immediately stored on ice. The cellular lysates were then treated for 2 h with1µl of proteinase K 20 mg/ml at 45°C and 1µl of RNase-A 10 mg/ml for 1hr at 37°C. Finally, the DNA samples were run in a Tris-Acetate-EDTA 2% agarose gel and visualized by staining with ethidium bromide.

Lactate dehydrogenase (LDH) assay

LDH is a cytoplasmic enzyme not released during active cell death like apoptosis, paraptosis, or autophagy, but released in necrosis due to the early increase of plasma membrane permeabilization. The LDH assay was performed according to the manufacturer's instructions of the LDH Cytotoxicity Detection Kit (Roche Applied Sciences). Following GCs treatment, an aliquot of culture supernatant was immediately stored on ice. Cells incubated with 1% Triton-X100 represented the positive control (100% of LDH release).The enzymatic reaction was initiated by the addition of 100 µl of reaction mixture (provided in the Kit) with 100 µl of supernatant during 30 min at room temperature. Reaction products were assessed spectrometrically at an absorbance of 490 nm *versus* 630 nm in 96 multi-well plates. The percentage of lysed cells was calculated using the following formula: 100× [(experimental LDH release)/(maximum LDH release)]. The maximum of LDH release corresponding to the amount of LDH released by a total lysis of the cells with Triton-X100. These experiments were performed 4 times.

Indirect Immunofluorescence experiments

Cells were seeded at density of 5.10^4 cells in 1 ml of complete medium in Lab-Tek devices (4 wells, Glass slide). Confluent cells were treated with LC_{50} of GCs at the indicated concentrations according to the cellular origin. The following proteins were tested: Light Chain 3 (LC3), cleaved Caspase-3, Apoptosis Inducing Factor (AIF) and Leukocyte Elastase Inhibitor/ LEI-derived DNase II (LEI/L-DNase II). At the end of the treatments, cells were washed three times with PBS (Phosphate Buffer Saline), fixed with 4% paraformaldehyde for 15 min at room temperature, washed three times in PBS, permeabilized with 0.3% de Triton-X100 for 15 min, washed three times with PBS for 5 min and then saturated with 1% BSA

(Bovine Serum Albumin) in PBS for 1 hr. Slides were then incubated with the polyclonal antibody against LC3 (Santa Cruz Biotechnology, sc-16756) and monoclonal antibody against AIF (EPITOMICS, ab32516) diluted 1:100 in PBS-0.1% BSA, overnight at 4°C, washed five times with PBS, incubated with the secondary antibodies: anti goat (Invitrogen, A11055, Alexa Fluor®) and anti-rabbit (Invitrogen, A10040, Alexa Fluor®) diluted 1:200 in PBS and incubated for 1 h at room temperature in a humidified chamber. Cells were then washed three times with PBS in the dark and finally mounted with Fluoromount (Sigma Life Sciences). For LEI/L-DNase II cell labeling was performed as described before (Altairac, *et al.* 2003). For cleaved Caspase-3 labeling, after fixation and permeabilization performed as above, cells were washed three times with PBS and then saturated with 1% skim milk in PBS for 1 hr. Cells were then incubated with monoclonal antibody against active caspase-3 (Cell Signaling, 9661S) diluted 1:100 in 0.1% skim milk PBS, overnight at 4°C, washed five times for 5 min with PBS, incubated with the secondary antibody (Invitrogen, A10040, Alexa Fluor® anti-rabbit) diluted 1:200 in PBS, for 1 h at room temperature in a humidified chamber. Cells were then washed three times with PBS in the dark and finally mounted with Fluoromount (Sigma Life Sciences). Cells were observed using a fluorescence microscope Olympus BX51, equipped with 40x and 60x objectives. Confocal analysis was done using a Zeiss LMS710 microscope. Adobe Photoshop was used as elaborating software.

Cytoplasm and nucleus fractions

Cells were collected by trypsinization and rinsed twice in PBS, then, 2.10^6 cells were resumed in 500 µl of a hypotonic solution of $MgCI_2$ (1.5 mM) during 10 min. Cells were then crushed with a Dounce potter and centrifuged 10 min at 390 g. The cytoplasmic fraction contained in the supernatant was removed and the nuclear fraction was resuspended in 1 mL of $MgCI_2$ (1.5 mM) and centrifuged during 10 min at 390 g twice, then resuspended in 200 µl of 10 mM Tris, 60 mM NaCl, 200 mM sucrose pH 7,4.

To measure the concentration of proteins in both cellular fractions, 10µl of the cytoplasmic and nuclear fractions are mixed to 250 µl of reactive solution (Pierce® BCA Protein Assay Kit, 23225) in a 96 multiwells plate. The plate was incubated 30 min at 37°C then the optical density was read in BioRad Benchmark plate reader at 595 nm. The obtained measures were correlated to a standard curve of BSA made in duplicate (0 to 1200 µg/µl). The sample concentration was calculated by linear regression.

Western blot analysis

Cells were disrupted in RIPA buffer (50 mM Tris, 150 mM NaCl, 1% Triton-X100, 1% Sodium Deoxycholate, 1% Sodium Dodecyl Sulfate) or M-PER buffer (Thermo Scientific, 78503) in the presence of protease and phosphatase inhibitors. The protein concentration was determined by the BCA method (as previously described). The proteins were distorted in Laemmli (62 mM Tris-HCI 6.8 pH, 2% SDS, 10% glycerol, 5% ß-mercaptomethanol, 0.01% Bromophenol blue). Equal amounts of protein (20 µg) were separated on a 12% Tris-Glycine gel. Nitrocellulose membrane transfer was performed at 100 V for 1:30 h at 4°C and monitored by the membrane staining with Ponceau Red (Sigma-Aldrich). The membrane was saturated with 5% skim milk in PBS for 1 h at room temperature then incubated overnight at 4°C with the primary antibodies against caspase-3 (1:1000) (Santa Cruz,1224), cleaved caspase-3 (1:1000) (Cell Signaling,9661), AIF (1:1000) (Santa Cruz, 9416), LEI/L-DNaseII (1:1000) (Torriglia, *et al.* 1995), β-Actin (1:1000) (Santa Cruz, 1616), Lamin B (1:1000) (Santa Cruz, 6216), LC3-II (1:1000) (Santa Cruz sc-16756). After 5 washes in PBS containing 0.1% Tween-20, the membrane was incubated for 1 hr with the anti-rabbit (Victor Laboratories, PI-1000) or anti-Goat (Victor Laboratories, PI-9500) secondary antibodies conjugated to peroxidase, diluted 1:5000, in PBS containing 0.5% Skim Milk and then washed 5 times in PBS. PARP (Poly ADP-Ribose Polymerase) western blot was performed as already described by Blenn and al (Blenn, *et al.* 2006). Visualization of the immunoreactive

bands was obtained by a chemoluminescent substrate, SuperSignal West Pico Chemiluminescent Substrate (Thermo scientific, 34087) using a MicroChemi 4.2 DNR BioImaging System.

Retinal flat mounting

After GCs treatment, retinas were immediately washed and fixed with 4% paraformaldehyde for 15 min. They were then permeabilized with PBS containing 0.1% Triton X-100, and incubated with the rabbit anti-caspase-3 and mouse anti-von Willebrand antibody or the chicken anti-LEI and rabbit anti-von Willebrand (1:100) overnight at 4°C. After washing 5 times with PBS, retinas were incubated with the secondary antibody (Alexa Fluor® 405-conjugated goat anti-rabbit IgG and Alexa Fluor® 594-conjugated Donkey anti-mouse IgG or Alexa Fluor® 568-conjugated goat anti-chicken IgG and Alexa Fluor® 405-conjugated goat anti-rabbit IgG (1:200)) for 1 hr. For phosphatase treatment, retinas were incubated for 30 min at 37°C with calf intestinal alkaline phosphatase (CIAP, Invitrogen, 8009-019) in dephosphorylation buffer (Invitrogen, P/N 01371) then washed 3 times. The dephosphorylation has as objective to unmask 3'OH DNA extremity stemming from activation of L-DNase II. Finally, Retinas were fixed with 4% paraformaldehyde then incubated for 1 hr with 80 µl of terminal deoxynucleotidyl transferase dUTP nick end labeling assay (TUNEL, Roche Diagnostics, Mannheim, Germany) reaction mixture at 37 °C. The retinas were flat mounted using Fluomount. Images were taken using a confocal laser scanning microscope Zeiss LSM 710 (Oberkochen, Germany).

Statistics

Data were expressed as means ± SE. Statistical analysis was made using the Graphpad Prism5 program (Graphpad Software, San Diego, CA, USA). Mann-Whitney test comparison was used. $P < 0.05$ deemed significant, unless otherwise stated.

Results

Dose-dependent effect of glucocorticoids (GCs) on microvascular endothelial cells viability (HMEC, BREC) and lethal concentrations (LC_{50}) determination:

In order to evaluate the sensitivity of HMEC and BREC to corticosteroids, cells were treated with different concentration of Hydrocortisone (Hydro), Triamcinolone Acetonide (TA), Dexamethasone (Dexa) and Dexamethasone Phosphate (Dexa-Ph) for 24h. Because hydrophobic glucocorticoids (Hydro, TA, Dexa) form dense and adherent toxic aggregates when directly added to culture medium (Szurman, *et al.* 2007), they were first dissolved in Ethanol 100%, then in the culture medium. This gave a final concentration of 1% ethanol in the medium. The reduced viability measured by the MTT assay was correlated to a reduced number of living cells, as verified by the trypan blue assay (not shown).Fig 1 shows that GCs had different concentration–dependent reduction of cell viability, depending on the GC and the cell type. Dexa-Ph was the less toxic and HMEC were more sensitive than BREC cells.

The mechanisms of cell death induced by GCs:

In order to analyze the mechanisms underlying GC toxicity on endothelial cells, we successively evaluated markers for autophagy, necrosis, caspase-dependent and caspase-independent apoptosis. These analyses were performed on HMEC using for each steroid its LC_{50} as determined above in order to have results obtained with the same degree of toxicity.

Autophagy

The presence of autophagy was analyzed using an antibody against MAP-LC3 (*microtubule-associated protein 1 light chain 3*). This protein is integrated to the autophagosomes membranes (Tanida, *et al.* 2004). In physiological conditions, the quiescent LC3 has a diffuse distribution in the cytoplasm. Once the autophagy activated, LC3 concentrates in the

autophagosome membrane and gives a punctuate labeling as observed in cells cultured in a depleted amino-acid (AA) medium, used as a positive control (Fig 2A). No such labeling was observed with GCs treatment eliminating autophagy as a potential mechanism of cell death (Fig 2A). This result was confirmed by western blot. As seen in Fig 2B only in AA depleted medium the lipidated form of LC3 (LC3 II) appeared.

DNA damage and necrosis

The DNA fragmentation into oligonucleosomal fragments is a widely recognized hallmark of apoptotic cell death, as induced by HMA (5-N, N-hexamethylene-amiloride) (used as a positive control). The "DNA ladder" pattern was clearly seen in samples treated with Dexa and Dexa-Ph, faintly with TA treatment and not at all in Hydro treated cells (Fig 2C). Moreover, as some smear degradation is also seen we investigated if necrosis was involved. This was done by measuring the release of LDH in the culture medium. A total lysis of the cells induced by 1% Triton-X100 was used as a positive control. Amongst the GCs tested at their respective LC_{50} concentrations, only Dexa (255 µM) induced a significant increase of LDH release (31.17%; $^{**}P \leq 0.01$) as compared to the positive control (Fig 2D).

Caspase-dependent apoptosis in GCs toxicity

Caspase-3 activation was analyzed by immunocytochemistry (Fig 3A) and western blot (Fig 3B). Amongst the GCs tested at their respective LC_{50} concentrations, only Dexa and Dexa-Ph induced activation of caspase 3 as observed on immunocytochemistry (Fig 3A). This was confirmed by western blot (Fig 3B). Only Dexa and Dexa-Ph induced the cleavage of pro-caspase 3. Moreover, PARP-1, one of the main targets of activated Caspase-3 was cleaved in cells treated with Dexa and Dexa-Ph but not in cells treated with other CGs (Fig 3B). In all these experiments the treatment of cells with etoposide, an inhibitor of toposiomerase II,

knowing to induce caspase dependent cell death was used as a positive control. The involvement of caspases in cell death was evaluated using the pancaspase inhibitor ZVAD-FMK. Results are seen on Fig 3C. The inhibitor successfully inhibits cell death in etoposide but not in GCs treated cells, suggesting that this pathway is not the main effector in GCs cells demise.

Caspase-independent apoptosis in GCs toxicity

Due to the lack of caspase activation in TA and Hydro, as well as the lack of protection with pancaspase inhibitor, we analyzed caspase independent pathways, namely Leukocyte Elastase Inhibitor (LEI/L-DNase II) and Apoptosis Inducing Factor (AIF).

LEI (Leukocyte Elastase Inhibitor) is a cytoplasmic molecule owning an anti-protease activity. After induction of apoptosis it is cleaved and transformed into L-DNase II, a molecule that has an endonuclease activity and that is translocated to the nucleus (Padron-Barthe, *et al.* 2007).

The activation of L-DNase II was estimated by immunostaining and Western blot. L-DNase II activation and nuclear translocation were observed with all GCs (Fig 4A). In order to verify the nuclear translocation of this protein we fractionated cytoplasms from nuclei, and performed a western blot on both fractions (Fig 4B). We observed the presence of native LEI (42 KDa) in the cytoplasmic fractions while the nuclear fractions contained, in addition, one band of 35kDa corresponding to the active L-DNase II (Belmokhtar, *et al.* 2000).Taken together these results indicate that GCs activate the LEI/L-DNase II pathway. HMA treated cells were used as positive control of L-DNase II activation.

Concerning AIF, (Fig 5) HMEC cells treated with the GCs presented a cytoplasmic dotted labeling which was compatible with its classical mitochondrial localization. This localization did not seem modified with the GCs (Fig 5A). Observe that staurosporine

treated cells used as positive control of AIF activation showed a diffuse cytoplasmic labeling and a nuclear peripheral labeling. As previously done, we verified this result by western blot (Fig 5B) after nuclear-cytoplasmic fractionation. The activated, cleaved form of AIF is only seen in staurosporine treated cells. These results indicate that the AIF is not activated by GCs in HMEC cells.

Effect of glucocorticoids on BREC

To verify if the cellular phenomena induced by the GCs on the HMEC were reproducible on retinal endothelial cells, we analyzed the activation of the cell death pathways previously studied.

For this purpose, BREC were treated for 24 h with $LC_{50,}$,then the activation of Caspase-3, LEI/L-DNase II and AIF were estimated by immunostaining with the corresponding antibody. The activation of Caspase-3 was observed on BREC treated with Dexa and Dexa-Ph, as already indicated for HMEC. Hydro and TA did not induce the caspase dependent cell death (Fig 6C). In same way, the activation of the LEI and nuclear translocation of L-DNase II were observed with all GCs on BREC (As showed in higher magnifications) (Fig 6A).The analysis of the immunocytochemistry of AIF on BREC showed a cytoplasmic dotted labeling indicating the mitochondrial localization of AIF with all GCs treatment, while cells treated with the staurosporine (positive control) revealed a diffuse labeling in the cytoplasm and a nuclear translocation (Fig 6B).

Impact of GCs on the retinal microvasculature

The *in vitro* experiments described above showed the toxic effects of GCs on HMEC and BREC. In order to be closer to physiological conditions, where the endothelial cells are organized as microvascular networks**,** rat retina explants were treated with GCs for 24 h at the

LC_{50} determined for BREC cells. An "*ex vivo*" model was preferred to a "*in vivo*" approach in order to better control the concentration of GCs reaching the retinal vascular bed. Actually, the intravitreal administration of drugs in rodents, due to the size of lens, which is relatively big, causes an inhomogeneous distribution of the injected substance and an uneven concentration on the different parts of the retina. The retinal microvasculature was immunostained with anti-Von Willebrand antibody. The double immunolabeling anti-caspase with TUNEL assay or anti-LEI/L-DNase II with TUNEL+Phosphatase assay allowed us to analyze the activation of these cell death pathways. As for HMEC and BREC, LEI/L-DNase II was activated on retina microvascular cells with all tested GCs as observed in our *in vitro* experiments (Fig 7A). The activation of Caspase-3 was observed in Dexa and Dexa-Ph treatments, while Hydro and TA did not induce a caspase-dependent cell death (Fig 7B).

Discussion

Intraocular glucocorticoids (GCs) are widely used to treat retinal diseases, directly injected into the vitreous as a non-soluble crystal suspension (like Triamcinolone Acetonide (TA)) or more recently as polymeric poly-lactic-co-glycolic acid (PLGA) implants releasing Dexamethasone (Dexa). The choice of the glucocorticoid is empirical since very few studies have compared their potential toxic and beneficial effects. In this study, we have focused on the potential toxicity of GCs on the microvasculature. We have focused on established microvasculature rather than in neovascularization which was largely featured in previous studies. Of course, no cellular model recapitulate a clinical condition or an established tissue and extrapolation should be performed with caution. In our experiments, we have used confluent cells, so that our experimental conditions are closer to established vessels than to proliferating vessels. In retinal explants, we have evaluated resting vessels instead of choosing

a model of neovascularization. In this study we have compared two cell lines of dermal and retinal origin in order to investigate differences in toxicity and we have then evaluated in detail the mechanisms of different GCs toxicity.. Our results show that endothelial cells originating from the retina (BREC) are less sensitive to all the GCs tested than endothelial cells from dermal origin (HMEC). Although the cells are from different origins in terms of species and the cells have different culture conditions, various factors concerning the tissue origin of the cells could explain this difference.. The first factor could be the natural microenvironment of these cells. In the eye, cortisol levels are higher than in the circulation and the corticoid-binding globulin is absent, explaining in part the immunologic privilege of the intraocular media (Denniston, *et al.* 2011). Moreover, in inflammatory conditions, as shown in experimental model of uveitis, cortisol levels increase in the ocular media (Bousquet, *et al.* 2012). This permanent exposure to high cortisol levels associated with the low proliferating capacity of retinal endothelial cells may explain their higher resistance to GCs-induced cell death (Joussen, *et al.* 2007).

Although the tested drugs belong to the same pharmacological family, they show different cellular toxicity. Their differential effects could be related to their relative affinity for gluco or mineralocorticoid receptors. Indeed, GCs can exert beneficial or deleterious effects on vascular permeability through junction adhesion and cytoskeleton remodeling (Chen, *et al.* 2002). However, the concentration of GCs used here is largely over the binding constant for these receptors (in the µM range) suggesting that the toxic effects are receptor independent.

Concerning endothelial cell toxicity, our results show that the more hydrophilic GCs, namely, Dexamethasone phosphate (Dexa-Ph) is the less toxic than the more lipophilic, Hydro, Dexa, and TA. This difference may be explained, by hydrophilic GCs transferring less easily through the intact membranes, resulting in lower bioavailability in cells and thus less toxic. It is worth noting that the toxicity measured here is not related to the anti-inflammatory activity of these

compounds. Compared to Hydro, TA has 5 to10 fold greater anti-inflammatory activity, while Dexa has 20 to 40 fold greater activity. However, their LC_{50} are quite similar (255 µM for Dexa, 230 µM for TA)(Cohen and Jacquot 1981, Le Jeunne 2012).

TA is the most widely administered intraocular GC and is usually given in a formulation not specifically adapted to ophthalmological use, as it was originally designed for intramuscular and intra-articular administration. Studies on the toxicity of TA and/or its excipient (benzyliquealcohol), the crystalline or soluble formulations, led conflicting rather than conclusive results (Torriglia, *et al.* 2010, Valamanesh, *et al.* 2007). Indeed, toxicity of TA and its excipient was not found in certain studies (McCuen BW 2nd 1981, Narayanan, *et al.* 2006) while other works clearly established the toxicity of TA and/or the excipient in the eye, resulting in a disorganization of the retina layers, a loss of the choroid network density and a vasoconstriction of the retina blood vessels (Morrison, *et al.* 2006, Torriglia, *et al.* 2010, Valamanesh, *et al.* 2009)**.**

TA showed concentration and time dependent cytotoxicity on retinal pigment epithelium cells, glial cells and endothelial cells of the retina. This toxicity triggered a reduction in mitochondrial activity, and an activation of caspase-independent cell death mechanism (paraptosis in EPR cells, LEI-L-DNase II and AIF in retinal endothelial cells (Narayanan, *et al.* 2006), (Valamanesh, *et al.* 2007, Yeung, *et al.* 2003)). This may explain the results obtained by some authors that showed no caspase activation after 24 h of treatment with TA on primary human retinal microvascular and flat mounted rat retinas (Hartnett, *et al.* 2006). Hence, the assessment of cell death pathways is an essential step in estimating the safety or the toxicity of health products. We show here that different cell death mechanisms other that classical caspase dependent apoptosis are responsible for cell death induced by GCs and that the same mechanisms seen in cell culture were also seen ex-*vivo* (microvessels of retina). Moreover, the use of the pancaspase inhibitor, ZVAD on HMEC show a lack of protective

activity regarding all the GCs suggesting that caspases are not the main effectors in this cell death.

In this study the toxicity of several GCs was estimated and compared for the first time, on microvascular endothelial cells of different tissue origins. This toxicity was previously disregarded because the tests used were not suitable for evaluating caspase-independent cell death. Strikingly, the concentrations used in our experiments, that proved to be cytotoxic, were lower than those used clinically. For instance, 4 mg of TA are injected in the human vitreous to treat macular edema. This produces a concentration of 1mg/ml (approximately 2.3 mM) of TA in the vitreous (lasting up to 1 month) due to the slow clearance of drugs in this compartment. The vitreous humor levels measured by several authors after triamcinolone acetonide intravitreous injection, are higher than the LC_{50} found in both HMEC and BREC (around 4 mM as compared to 230 and 460 µM for HMEC and BREC respectively) (Oliveira, *et al.* 2012).

GCs have widespread clinical applications in the eye and in other organs and their use is often the only available therapeutic option. In this work we demonstrated that high concentrations of these compounds could be toxic to endothelial cells, of microvasculature and target tissues. It is essential to ensure their concentrations remain under the toxic threshold for endothelial cells when developing novel therapeutic protocols.

References

Legends

Figure 1: Evaluation of the toxicity and lethal dose (LC_{50}) of different glucocorticoids (GCs) on human endothelial cells (HMEC) or bovine retinal endothelial cells (BREC). Cells were treated with Hydrocortisone (Hydro), Triamcinolone Acetonide (TA), Dexamethasone (Dexa), Dexamethasone Phosphate (Dexa-Ph) for 24 h at the indicated concentrations. The lipophilic GCs (Hydro, TA, and Dexa) were dissolved in Ethanol 100% then in cell culture medium. Control cells were either exposed to 1% ethanol or were left untreated. Cell viability was evaluated using the MTT assay. (A) HMEC; (B) BREC The lipophilic GCs show a concentration-response relationship between the decline of viability and the increase of the dose while Dexa-Ph is less toxic. These are representative results of three different experiments.

Figure 2: **Cell death in GCs treated HMEC cells**. (A) The activation of autophagy process was evaluated by anti-LC3 immunocytochemistry. HMEC cells were treated for 24 h with the LC_{50} of GCs. For the positive control cells were incubated with medium lacking amino acids. HMEC cells were fixed, permeabilized and immunostained with anti-LC3 (green), all treatments revealed diffuse cellular labeling of LC3 confirming the absence of autophagic vesicles. Scale bars represent 5µm. (B) Cells treated as above were analyzed by western blot. The activation of autophagy in AA deprived cells triggers the appearance of a 14kDa band, LC3II, the lipidated form of LC3. (C) DNA extracted from HMEC treated with LC_{50} of GCs were analyzed by electrophoresis on a 2% agarose gel. Oligonucleosomal fragments of DNA are seen with Dexa, Dexa-Ph and slightly with TA. Control: Untreated cells, Ethanol 1%: medium containing 1% of ethanol, treatment with 40 µm HMA (5-N, N-hexamethylene-amiloride) was used as a positive control for apoptosis. (D) The release of lactate dehydrogenase (LDH) from HMEC cells treated 24 h with LC_{50} of GCs was measured in the

culture medium and compared to the maximal LDH release, determined by exposing the cells to 1% of Triton X-100. Each experiment was performed in triplicate. Data are expressed as medium percentage of LDH released into the media. Dexamethasone induced an increased release of LDH as compared to control and to the other treatments (**P≤0.01).This is a representative experiments out of 4.

Figure 3: The activation of caspases in GCs treated HMEC cells. (A) The activation of caspase-3 by immunolabelling of HMEC cells using an anti-active caspase-3 antibody (red). Cells were exposed for 24 h with the LC_{50} of GCs. Cells incubated with Etoposide were used as positive control. Only Dexa and Dexa-Ph induced an activation of caspase-3 (arrows). These are representative results out of 3 independent experiments. Scale bars represent 7 µm. (B) Caspase-3 and PARP western-blot analysis. Protein extracts from GCs treated HMEC cells were analyzed in a 12% acrylamide gel, transferred to nitrocellulose and revealed with anti-caspase-3. Apoptosis induced in HMECs with Etoposide (100 µM for 24 h) was used as positive control. Activated caspase-3 and cleaved PARP were detected in HMECs treated with Dexa and Dexa-Ph, showing that both GCs induce caspase-3 activation. Representative results of 3 experiments. (C) Effect of ZVAD on HMEC survival. Cells were exposed for 24 h with the LC_{50} of GCs in the presence (grey bars) or in the absence (white bars) of ZVAD-FMK. Cells incubated with Etoposide were used as positive control. MTT was used to evaluate cell viability. No protective effect is seen in GCs treated cells. Results were analyzed by the ANOVA test using the Newman-Keuls post test. *** indicates significant differences $p< 0.001$.

Figure 4: **Activation of the L-DNase II pathway in GCs treated HMEC cells**. (A) HMEC cells were treated with the LC_{50} of GCs for 24 h and immunostained with an anti-LEI/L-DNase II (red). HMEC Cells exposed to HMA represent the positive control. All the GCs led to LEI/L-DNase II activation with an intense nuclear staining. Scale bar represents 10µm. These are representative results out of 4 experiments. (B) GCs treated HMEC were fractionated into cytoplasmic and nuclear fractions. Western blot analysis using an anti-LEI/L-DNase II revealed an accumulation of L-DNase II in the nucleus of treated cells, phenomena also seen in HMA treated cells used as positive control.

Figure 5: **AIF activation in GCs treated HMEC cells.** (A) Nuclear translocation of AIF was evaluated by anti-AIF immunocytochemistry (green). HMEC cells were treated for 24 h with the LC_{50} of GCs, for the positive control cells were treated with Staurosporine (Stauro). Control cells and GCs treated cells revealed a dotted labeling of AIF. Scale bars represent 10 µm. These are representative images of 2 independent experiments performed in quadruplicate (B) AIF western blot was performed on nuclear and cytoplasmic fractions. AIF was present in all cytoplasmic fractions, only the Staurosporine treated cells, used as a positive control, show a nuclear translocation of the truncated (active) form of AIF. Representative experiment out of 2.

Figure 6: **The activation of caspase dependent and independent cell death in GCs treated BREC cells**. For all experiments BREC were treated during 24h with LC_{50} of GCs. (A) BREC cells were immunostained with an anti- LEI / L-DNase II. Cells exposed to HMA represent the positive control. All the GCs led to LEI/L-DNase II activation with an intense nuclear staining (white arrow heads) the same outcomes was observed previously with

HMEC. LEI/L-DNase II was stained in red, nuclei in blue. The right column represents confocal images of cells showing a nuclear translocation of L-DNase II. Scale bars represent 20µm for fluorescence images and 10µm for confocal images. (B) Nuclear translocation of AIF in BREC was evaluated by anti-AIF immunocytochemistry. Cells treated with Staurosporine represent a positive control. GCs treated cells revealed a dotted labeling of AIF, which suggests a mitochondrial localization. White arrow head shows a nuclear translocation of AIF. AIF is seen in green, nuclei in blue. Scale bars represent 20µm for fluorescence images and 10µm for confocal images.. (C) Activated caspase-3 immunocytochemistry of BREC cells. BREC were immunostained with an antibody that specifically recognizes the cleaved caspase-3. Etoposide was used as a positive control. Like previously observed with HMEC cells only Dexa and Dexa-Ph induced an activation of caspase-3. Activated caspase 3 is seen in red, nuclei in blue. Scale bars represents 20µm. All experience were performed at least 4 times.

Figure 7: **Cell death pathway induced by GCs on retinal microvasculature**. Retinas from Lewis rats were flat mounted, treated with GCs and analyzed. The retinas treated with steroids-free medium were used as control. (A) Retinal explants were treated with GCs, and immunostained for LEI/L-DNase II (red), TUNEL-Phosohatase (green), and won Willebrand factor (blue). L-DNase II was activated with all treatments. (B) Retinal explants were treated with GCs, and immunostained for caspase-3 (blue), TUNEL (green) and won Willebrand factor (red). The activation of Caspase-3 on retina vessels was induced with Dexa. Scale bar represents 30 µm. Each experiment was performed at least 4 times.

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