Choroidal mast cells in retinal pathology: a potential target for intervention

Elodie Bousquet[1, 2], Min Zhao[1], Brigitte Thillaye-Goldenberg[1], Viera Lorena[1], Beatriz Castaneda[1], Marie-Christine Naud[1], Ciara Bergin[3], Bernadette Besson-Lescure[4], Francine Behar-Cohen[1, 3]*, Yvonne de Kozak[1]*

[1] INSERM U1138, team 17 : Physiopathology of retinal diseases : therapeutic innovations, Université Sorbonne Paris Cité, Université Pierre et Marie Curie, Centre de Recherche des Cordeliers, Paris, France,

[2] Department ophthalmology Hôtel-Dieu of Paris, Assistance Publique Hôpitaux de Paris, Paris, France, Université Sorbonne Paris Cité

[3] Department of Ophthalmology of Lausanne University, Jules Gonin Ophthalmic Hospital, Lausanne, Switzerland

[4] Plateforme technologique de phénotypage du petit animal et microdosages, IFR65/IRSSA, Hôpital Saint-Antoine, Paris, France

*Both authors have contributed equally to the work

Number of text pages: 21
Number of figures: 7
Running head: mast cells in retinal pathology

This work was supported by grants from the National Institutes of Health and Medical Research, INSERM, by the Centre National de la Recherche Scientifique, CNRS, by the Association CRO, and by the Fondation pour la Recherche Medicale (for supporting Elodie Bousquet).

Corresponding author : Yvonne de Kozak INSERM U1138, Physiopathology of Ocular Diseases: Therapeutic Innovations, Centre de Recherche des Cordeliers, 15 Rue de l'Ecole de Médecine, 75270, Paris cedex 06, France. Phone: 33 1 44 27 81 64. Fax: 33 1 44 27 81 12.
E-mail: yvonne.de_kozak@crc.jussieu.fr

Abstract

We previously showed that mast cells are important in the initiation of ocular inflammation but the exact consequences of mast cell degranulation on ocular pathology have not been characterized. We induced mast cells depletion of their inflammatory mediators by the local sub-conjunctival injection of 48/80 drug. Initial degranulation of mast cells was observed in the choroid 15 min after the injection of 48/80, with degranulation continuing to increase up to 3-6 hrs after the injection. The signs of anterior segment clinical inflammation paralleled mast cell degranulation. Posterior segment imaging using optical coherence tomography showed increased choroidal thickness and serous retinal detachments, which were confirmed by histological analysis. The infiltration of polymorphonuclear cells was associated with increased ocular media levels of TNF-α at 1 and 3 hrs, followed by CXCL1, IL-6, IL-5, CCL-2 at 6 and 24 hrs, and IL-1β at 24 hrs. Analysis of levels of VEGF and IL-18 at all time intervals showed an opposite evolution of VEGF as compared to IL-18 levels suggesting that they could control each other production. These findings suggest that the local degranulation of ocular mast cells provokes acute ocular inflammation, increased choroidal vascular permeability and serous retinal detachments. The involvement of mast cells in retinal diseases should be investigated and the pharmacological inhibition of mast cells degranulation considered as a potential intervention target.

Introduction

Mast cells have been detected in the lids, conjunctiva, the uveal tract (iris, ciliary body and choroid) of several different species.[1,2,3,4,] In humans and rats, mast cells are abundant in the anterior and posterior uvea but absent in the retina.[5] These cells are understood to originate from hematopoietic progenitors and following migration to vascularized tissues to mature locally.[6] Mast cells are activated by danger signals which have a specific pathogen-associated molecular pattern; exogenous signals such as lipopolysaccharide (LPS), zymosan, and peptodiglycan[7] and endogenous signals such as Interleukin-33 (IL-33), a cytokine of the IL-1 family have been shown to cause activation.[8] It is understood that the fusion of secretory granules with the plasma membrane results in the quick release of a number of cytokines and vasoactive amines such as histamine, contributing to the recruitment of neutrophils.[9] As such, mast cells initiate immune responses, in cases such as allergic disorders and immune responses to pathogens.[6,10,11]

In the eye, degranulation of choroidal mast cells has been shown to contribute to the early onset of Experimental Autoimmune Uveoretinitis (EAU)[4,12] and of Endotoxin Induced Uveitis (EIU).[13,14] On the other hand, the depletion of mast cells from their inflammatory mediators with the compound 48/80 was shown to prevent EAU development.[12] Moreover, topical ocular medications designed to block mast cell degranulation have been reported to decrease the clinical severity of the disease during EAU.[12] Serous retinal detachments (SRD) which are almost always observed during EAU are often observed in eyes with ocular inflammatory disorders such as Vogt-Koyanagi-Harada disease.[15] However there may be multiple mechanisms leading to SRD, as they also are present in other ocular disorders such as central serous chorioretinopathy and wet Age related Macular Degeneration (AMD).[16] In AMD for example, genetic studies have demonstrated that complement alternative pathway activation is associated with increased prevalence of the disease.[17,18,19] Deposits of C5a, a potent mast cells degranulating component,[20] have been detected in the choroid and retinal pigment epithelium of eyes with AMD,[21] suggesting that mast cells may be a complement pathway effector in AMD pathogenesis.

In order to clarify the direct role of mast cells in ocular pathology, we examined the effects of specific ocular mast cells degranulation in rats by the local unilateral subconjunctival injection of the synthetic compound 48/80.

Materials and Methods

Animals

Adult female Lewis rats (6–8 weeks old, Janvier, Le Genest-Saint-Isle, France) were used in this study. Animals were housed in a 12-hrs light and 12-hrs dark cycle and fed water and dried ration ad libitum. Experimental procedures were submitted and approved by the ethic committee of Paris Descartes University (number: Ce5/2012/122). The care and use of the animals was in compliance with ARVO statement for the Use of Animals in Ophthalmic and Vision Research. Accreditation N° of the laboratory: B 75 06 02.

Injections

Anesthesia of rats was performed by intraperitoneal injection of pentobarbital (25 mg/kg Nembutal; Abbot, Saint-Remy sur Avre, France). One drop of 1% tetracaine (Sigma-Aldrich, Saint Quentin Fallavier, France) was instilled for local anesthesia.

-*Subconjunctival administration of compound 48/80:* One subconjunctival injection of solution of compound 48/80[12,14] (Sigma-Aldrich) (120 μg of 48/80 in 100 μL of sterile pyrogen-free saline), was performed in the right eye in the temporal site of the conjunctiva as previously described[12] using 1 mL syringes with 30G needle. Left eye was not injected.

-*Subconjunctival administration of NaCl*: Control rats were injected subconjunctivally with vehicle (NaCl 0.9%).

-*Subconjunctival injection of disodium cromoglycate:* Disodium cromoglycate (DSCG, cromolyn sodium salt, Sigma-Aldrich) blocks the release of mediators from mast cells.[6] One subconjunctival injection of DSCG (2 mg DSCG/rat weighing 200 g dissolved in 100 μL saline) was administered at the nasal site 15 min before the subconjunctival administration of compound 48/80 performed at the temporal site.

In vivo investigations

Clinical examination

Eyes were examined with a biomicroscope (slit lamp) at regular time intervals in 48/80-injected eyes, in contralateral non-injected eyes and in eyes from the group injected with DSCG i.e. 10-30 min, 1, 2, 3, 4, 5, 7, 24 hrs after 48/80 subconjunctival injection. At each time point, the conjunctiva, lens, iris and pupil and the anterior chamber were observed. Pupillary dilation was obtained after administration of a drop of mydriaticum (Thea, Clermont Ferrand, France). The severity of the reaction to the subconjunctival injection of 48/80 took into account each ocular inflammatory sign: -conjunctival edema, -vasodilation of iris vessels,

myosis, - fibrin exudate, - hypopion, -iris haemorrhages, - synechiae. Presence of each sign was encoded as 1, absence as 0, the severity of the response was evaluated in each eye by the sum of the pathological signs/eye. Alteration of the lens transparency (cataract) was also evaluated.

Optical Coherence Tomography (OCT)

The assessment of rat retina and choroid was performed on anesthetized animals using spectral domain optical coherence tomography (SD-OCT, Spectralis™ device, Heidelberg, Germany) adapted for small animal eyes.[22] Pupils were dilated with 5% tropicamide drops. Scans were taken in the same rats prior to treatment and at 1, 3, 6 and 24 hrs after 48/80 subconjunctival injection. Each two-dimensional B-scan recorded at 30° field-of-view consisted of 1536 A-scans with an optical resolution reaching 3.5 μm and the enhanced depth imaging option (EDI) was used to evaluate the choroidal thickness.[23]

Ex vivo investigations

Experimental groups of rat eyes were as follows: 48/80-injected eyes, contralateral non-injected eyes and NaCl-injected eyes, enucleated at 4 time points after 48/80 injection at namely 1 hr, 3 hrs, 6 hrs and 24 hrs. Number of experimental eyes are indicated in figure legends.

Flat-mounted preparations of the choroid (as previously described[4]) for staining and quantification of choroidal mast cells degranulation

-*Choroidal mast cell staining*: The retina was removed and the choroid and the adhering sclera were placed into a fixative solution containing: anhydrous acetic acid: 95% ethanol : 40% formalin : distilled water = 1 : 15 : 6 : 30. After 60 min, the choroid was detached from the underlying sclera, washed in 95% ethanol and stained for 40 min in 0.25% solution of toluidine blue in 70% ethanol acidified with HCL (pH 2.4). Choroids were dehydrated and mounted in Eukitt (VWR, Fontenay-sous-Bois, France). Under these staining conditions, only mast cell granules were stained.

-*Choroidal mast cell quantification:* Choroidal mast cells were then counted using an ocular grid at the magnification of x250, 200 to 1000 mast cells were assessed on 10 to 20 grid areas for each choroid. Mast cells were considered as degranulated when showing an irregular shape, altered cell membrane and extracellular granules.

The density of degranulated and non-degranulated mast cells (mean cells ± SEM per mm^2 of choroid) were calculated at different time intervals post-injection in 48/80-injected eyes and in contralateral non-injected eyes.

Immunohistochemistry on flat mounts of RPE/choroid

Enucleated eyes were fixed in 4% paraformaldehyde (PFA) for 15 min at room temperature and sectioned at the limbus; the anterior segment and the retina were discarded. Flat mounts of RPE/choroids were fixed for additional 15 min in acetone at −20°C. Specimens were incubated overnight at 4°C with primary antibody diluted in PBS and 0.1% Triton X-100. Primary antibody used was: rabbit polyclonal anti-occludin (dilution 1 : 200; Zymed, San Francisco, CA, USA). Occludin is an integral plasma-membrane protein located at the tight junctions and is involved in cell adhesion and permeability.[24] The corresponding Alexa secondary antibody (Invitrogen life technology Carlsbad) was used to reveal the primary antibody and sections were counterstained with 4', 6-diamino-2-phenylindol (DAPI) (Sigma-Aldrich). Sections and flat-mounts were viewed with a fluorescence microscope (Olympus BX51) and confocal microscope (LSM 510 laser scanning microscope Zeiss, Carl Zeiss, Le Pecq, France). Images were exported to Photoshop software for preparation of final images. Staining that omitted the primary antibody served as negative control.

Histological sections for the assessment of ocular pathology and choroidal thickness

Eyes were fixed in 0.5% glutaraldehyde / 4% paraformaldehyde (PFA) for 2 hrs, dehydrated in a graded alcohol series (50%, 70%, 95% and 100%) and embedded in epoxy resin, 5 μm section were cut and stained with toluidine blue. The morphology and pathological modifications of ocular tissues were examined under a light microscope (DM5500B, Leica, Nanterre, France).

Choroidal thickness was measured (ImageJ) on photographs taken on historesin sections from the optic nerve to the ciliary body. Each measurement was taken at 300 μm on each side of the optic nerve[25] of 48/80-injected eyes, contralateral non-injected eyes and NaCl-injected eyes.

TUNEL assay

Enucleated eyes were snap frozen in Tissue-Tek OCT-compound (Bayer Diagnostics, Puteaux, France). Cryostat sections (10μm) were fixed in 4% PFA for 15 min and permeabilized with 0.1% Triton X-100 for 30 min. Apoptosis of cells was assessed on

cryostat sections with a terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) kit (Roche, Indianapolis, IN) in strict accordance with the manufacturer's instructions.

Cytokine and chemokine analysis in ocular fluids by multiplex ELISA

Aqueous humor and vitreous body (ocular media) from each eye were collected, pooled and centrifuged allowing separation of ocular fluids from ocular infiltrating cells. According to the manufacturer's instructions, multiplex ELISA assay (Milliplex Map Kit, Saint-Quentin en Yvelines, France) was performed on rat ocular fluids (xMAP technology assay) as follows: the following inflammatory mediators were measured: chemokines: MCP-1/CCL2, MIP1-α/CCL3, RANTES/CCL5, IP10/CXCL10 (IFN-inducible protein-10) and GRO/KC/CXCL1/ CINC; inflammatory mediators: IL-1β, IL-18, TNF-α, IL-2, IFN-γ, IL-4, IL-5, IL-6, IL-10, IL-13, IL-17 and VEGF (Clinisciences, Montrouge, France). Detection thresholds were estimated around 1 to 10pg/mL. The nomenclature CCL2 and CXCL1 was used throughout the study.

Statistics

Data were expressed as means ± SD. Statistical analysis was made using the Graphpad Prism5 program (Graphpad Software, San Diego, CA, USA). A Mann Whitney test was used for comparison between two groups and comparisons more than two groups were performed using one-way ANOVA test followed by Bonferroni's comparison, significance was investigated at the 5% level for all test types.

Results

Subconjunctival injection of 48/80 induces clinical ocular inflammation

Biomicroscope (slit lamp) examination was performed at different time intervals after 48/80 or saline injection. Unilateral clinical signs of ocular inflammation were observed (Fig. 1A, B). At 10 min post 48/80 injection, conjunctival edema and intense vasodilation of iris vessels were observed (Fig. 1A). From 3 hrs to 24 hrs, hazy cornea, fibrin deposit, hypopion (small amount of pus or collection of white cells) and synechiae (adherence of the iris to the lens) were observed in the anterior chamber of the eyes. Punctate hemorrhages in the iris were occasionally found (Fig. 1A). In 17 out of 25 rats (68%) a transitory cataract was observed from 1hr post 48/80 injection, that persisted until the 3^{rd}-6^{th} hrs and then faded. No clinical

sign of inflammation could be observed in contralateral non-injected eyes and in rats injected with NaCl (not shown).

As shown in Fig. 1B, whereas no pathological signs were detected in the left eyes not injected with 48/80, all right eyes injected with 48/80 showed significant signs of anterior segment inflammation from 15-30 min reaching a peak at 1-2 hrs and significantly slowly decreasing between 3 and 24 hrs post injection.

In the eyes where mast cell mediator release inhibitor DSCG was injected 15 min prior to 48/80, no clinical signs of inflammation were observed (not shown).

Morphological modifications and quantification of mast cells in choroid flat mounts.

The morphology of choroidal mast cells was analysed using flat mounts preparations (Fig. 2). Mast cells are regularly located around large choroidal vessels. In left non-injected eyes (Fig 2 A-C, inset) and NaCl-injected eyes (not shown), mast cells displayed an intact morphology at all-time points with high number of condensed intracellular granules intensively stained with toluidine blue in the cytoplasm. In contrast, as early as 15 min after 48/80 injection occasional degranulated mast cells were seen (not shown). At 1 hr after 48/80 injection, the number of degranulated mast cells importantly increased with granules extruded from the cytoplasm (Fig. 2 D, inserts). At 3 hrs, mast cells showed reduced density of cytoplasmic granules and an extensive and massive degranulation in all mast cells present in the choroid (Fig. 2 E, insert). At 24 hrs, all mast cell still presented an abnormal shape but the granules were again stained intensively (Fig. 2 F, insert).

Choroidal mast cells density was calculated on flat mounted preparations. Variations in the number of choroidal mast cells (degranulated or intact) at 1h and 3h are shown in Fig. 2 G, H. Compared to contralateral non-injected eyes, the number of degranulated mast cells was significantly higher at 1 hr and 3 hrs in 48/80 injected eyes (Fig. 2 G). Correlatively, significantly lower number of non-degranulated mast cells was found in 48/80-injected eyes compared to non-injected eyes (Fig. 2 H).

Ocular pathology induced by mast cells degranulation

Pathological modifications of ocular tissues were evaluated in historesin sections. In left non-injected eyes, mast cells showed a normal resting shape (Fig. 3, A, C), at the vicinity of vessels at the limbus of the cornea (Fig. 3 A, insert) and also in the choroid (Fig. 3 C, insert). In the right 48/80-injected eyes, at 1 and 3 hrs, degranulated mast cells were observed in the vicinity of vessels at the limbus of the cornea (Fig. 3 D, insert) and in the conjunctiva (Fig. 3

E, insert). In these eyes, mast cell degranulation in the anterior segment was associated with intense infiltration of PMNs (Fig. 3 F, insert, arrowhead) and macrophages (Fig. 3 F, insert, arrow) at 3 hrs to persist up to 24 hrs, and with alterations of corneal endothelial cells (Fig. 3 F, asterisk). In the posterior segment, mast cell degranulation was associated with fibrin exudate above the RPE in the subretinal space (Fig. 3 G, arrow) together with choroidal vessel dilation (Fig. 3 G, asterisk). Moreover, exudative retinal detachments appeared in the same locations as moderate to extensive ruptures of the RPE barrier and important retinal folds (Fig. 3 G and 3 H).

Starting at 1 hr (Fig. 3 G) after 48/80 injection to be very important at 3 hrs-24 hrs (Fig. 3 H), alterations of the retinal nuclear cell layers with picnotic aspects of the nuclei of photoreceptors and bipolar cells visible at the level of the retinal detachment. At 24 hrs, detachments and large destructions of the retina were observed (Fig. 3 H). Numerous TUNEL-positive nuclei were detected at 3 hrs (Fig 3 J) and at 24 hrs (Fig. 3 K), in the three nuclear cell layers, aspects consistent with the pathological aspect of retinal cells shown in historesin section (Figure 3 H).

OCT examination of posterior segment of the eye

One hour post 48/80 injection, the retina presented undulations, the apex of which were hyper reflective (Fig. 4 A, B, arrows). From 3 hrs to 24 hrs after 48/80 subconjunctival injection, larger and multiple retinal serous detachments were observed (Fig. 4 D, E, G, F) with pockets of subretinal fluid (Fig. 4 D, E, G, F, stars). Detachments of the neuroretina, due to subretinal accumulation of material were observed on histological section (Fig. 4 C, arrow). Edema was located in the retinal nuclear cell layers (Fig. 4 C, F, arrowheads). An increase of the choroidal thickness (Fig. 4 H, I, arrowheads) was observed related to dilation of the choriocapillaris (Fig 4 I star) and large choroidal vessels (Fig 4 I asterisk). No pathological changes were observed in contralateral non-injected eyes.

Increase of choroidal thickness in 48/80-injected eyes

At 1-3 hrs, compared to choroidal thickness in the left non-injected eyes and in NaCl-injected eyes, a significant and localized increase of choroidal thickness was detected in right 48/80-injected eyes (Fig. 5 A, B). At 6 -24 hrs, the choroidal thickness increase extended also to the periphery and all over the choroid surface (Fig. 5 C, D).

RPE barrier breakdown induced by mast cell degranulation

RPE flat mount were used to visualize the RPE cells junctions at 1 hr after 48/80 injection. In NaCl and contralateral non-injected eyes, occludin labelled a regular hexagonal membrane of the RPE cells (Fig. 4 A). In 48/80-injected eyes, an abnormal shape in some RPE cells with irregular occludin labelling (Fig. 4 B, arrowhead) was observed. In particular RPE cells defects (Fig. 4 B, C, stars) and localized enlargement of intercellular space (Fig. 6 D, arrowhead) were present throughout.

Close examination of historesin sections, showed a regular interface between the RPE cell monolayer and Bruch's membrane in contralateral non-injected eyes (Fig. 4 E, arrows), whereas in 48/80-injected-eyes, large alterations of the RPE were detected at all time points, in locations adjacent to the choriocapillaris (Fig. 6 F asterisk). RPE cells detached from the basal membrane and migrated, in either isolated or as large sheets of RPE, into the subretinal space (Fig. 4G, arrowhead). These round, binuclearly detached cells, allowed the positive identification of the RPE cells (Fig. 4 G).

Effect of mast cells degranulation by compound 48/80 on ocular cytokine and chemokine concentrations in ocular fluids

Multiplex ELISA analysis showed that the level of several inflammatory mediators was significantly higher in 48/80-injected eyes compared to control NaCl-injected eyes (Fig. 7). As early as 1 hr after 48/80 injection, TNF-α was significantly increased as compared to NaCl-injected eyes (Fig. 7 A). This increase persisted at 3 hrs, but not at 6 hrs. A significantly increased level of IL-5 was observed at 3 hrs, 6 hrs and 24 hrs (Fig. 7 B), together with significantly increased levels of IL-6 and CCL-2 at 6 hrs and 24 hrs (Fig. 7 C, D). Furthermore a significant increase of IL-1β, CXCL-1 and IL-18 was observed at 24 hrs (Fig. 7 E, F, G) while significant decrease of VEGF occurred at this time (Fig. 7 I). Levels of IL-18 (Fig. 7 H) and VEGF (Fig. 7 J) mirrored each other suggesting co-regulation. The cytokine levels in the contralateral non-injected eyes were consistent with levels found in NaCl eyes.

Discussion

A local subconjunctival injection of 48/80 (which specifically induces mast cells degranulation), was used to analyse the sequence of events occurring in the eye after mast cell degranulation. It showed a strong clinical and morphological inflammatory reaction detected in both posterior and the anterior segments of the eye. But the acute mast cell degranulation in ocular tissues also induced remarkable posterior segment pathology, beginning with choroidal

thickening and outer retinal barrier breakdown that resulted in serous retinal detachments (SRD). These SRD are similar to those observed in some forms of acute posterior uveitis such as Behçet[26] and Vogt-Koyanagi-Harada[15] diseases, indicating that these eyes may share some pathogenic mechanisms. Since mast cells mature locally in the vicinity of vascularized tissues, the site of their final residence,[6] a local degranulation of mast cell in one eye is not expected to induce pathology in the contralateral eye, which was confirmed by unilateral clinical signs and the lack of morphological changes in the contralateral eye.

Interestingly, a second subconjunctival injection of 48/80 performed 7 days after the first one, induced the same pathological signs (not shown). In human pathology, one could expect that if the stimulus persists, successive degranulations could occur, leading to chronic signs.In 48/80-injected eyes, mast cell degranulation elicited a complex sequence of morphological changes. The early blood-aqueous barrier breakdown, coincided with a transitory cataract, which dissipated spontaneously by 6 hrs post 48/80 injection. Such transitory cataract has not been observed in other acute models of intraocular inflammation, suggesting that mediators specifically released by mast cell degranulation induced metabolic alterations of the lens epithelium and /or disruption of epithelial tight-junctions, altering the lens homeostasis and inducing lens opacity.[27] In addition to the anterior segment inflammation induced by 48/80, choroidal mast cells degranulation provoked posterior segment pathology. As early as one hour post 48/80 injection, serous retinal detachments (SRDs) were observed. These SRDs increased in severity (area and height) with time up to 24 hrs as observed with *in vivo* OCT imaging. At this time point, anterior segment inflammation was observed to lessen. On histology, choroidal thickening and choroidal vessels dilation were observed very quickly post 48/80 injection, followed by RPE barrier disruption and SRD, which were associated with numerous RPE cells defects. The notably short time from mast cells degranulation until RPE barrier breakdown suggests direct junction destabilization by the released mediators. Histamine has been shown to induce tight-junctions opening in corneal endothelial cells[28] and in retinal vascular endothelium.[29] Histamine administered intravenously also increased choroidal blood flow as evaluated by laser doppler in humans.[30] But whether histamine induces direct effects on RPE tight-junctions, or whether it is a consequence of other inflammatory mediators has not yet been explored. In rat EAU, we have shown that the degranulation of choroidal mast cells peaked before the first pathologic signs were observed, suggesting the role of early mast cell degranulation as a trigger of ocular edema, inflammation and lesions.[4,12] Other inflammatory mediators released by mast cells, together with ocular infiltrating cells, may also participate to induce tissue lesions.[4,13,14]

Mast cells have a large catalogue of cell surface receptors, such as IgE receptors, complement component receptors, and various Toll like receptors. Moreover, mast cells respond to a large variety of stimuli[31] and induce the synthesis of TNF-α and IL-6.[32] In the present study, TNF-α, was the first cytokine to be up regulated in the eye (1 hr after 48/80 injection), followed by IL-6 (6 hrs), confirming that TNF-α could be an inducer of IL-6 release during ocular inflammation.[33] It is understood that TNF-α can contribute to the outer retinal barrier breakdown, as rat RPE cell monoculture co-incubated with TNF-α together with LPS+IFN-γ altered RPE tight junctions with abnormal distribution of ZO-1.[34] Experimental ocular inflammation is characterized by the breakdown of blood-ocular barriers allowing inflammatory cells, including macrophages and polymorphonuclear leukocytes (PMNs), to invade the ocular tissues (Protein Kinase C_ (PKC_) Regulates Ocular

Inflammation and Apoptosis in Endotoxin-Induced Uveitis (EIU) Signaling Molecules Involved in EIU Resolution by PKC_Inhibitor and Interleukin-13. The American Journal of Pathology, Vol. 170, No. 4, April 2007). Mast cells also modulate neutrophil influx and recruit neutrophils through TNF-α release.[35] The injection of HrIL-6 (Human recombinant IL-6) into the vitreous has been reported to induce an accumulation of neutrophils in the anterior and posterior segments of the eye.[36] Further, in the present situation, the rat chemokine CXCL-1 (a neutrophil chemoattractant) was also significantly increased and responsible, together with TNF-α and IL-6, for the neutrophils infiltration of the anterior chamber. Combined, these different mediators, successively produced, created the structure to allow the neutrophil infiltration observed after 48/80 injection in study eyes. Further, IL-18, a potent neutrophil-activating factor,[37,38] was augmented after 48/80 injection with an opposite regulation of the levels of VEGF. At 24 hrs, significantly higher levels of IL-18 and lower levels of VEGF were observed in 48/80-injected rats as compared to contralateral eyes. The exact role of IL-18 in the eye is still unclear[39] but VEGF and IL-18 were shown to suppress each other's production.[40] In a mouse model of neovascularization,[41] IL-18 inhibited new vessel formation but promoted damages to the RPE that mimics aspects of the dry form of AMD.[42] Alteration of RPE cells after mast cell degranulation could thus be at least in part related to IL-18 increase.

This study shows for the first time that mast cells degranulation induces choroidal, RPE and retinal pathology resulting in serous retinal detachment, a sign common to various retinal diseases. Development of spectral-domain OCT using enhanced depth imaging module has allowed to identify a wide spectrum of chorioretinal diseases associated with pachychoroid

(choroidal thickening) such as central serous chorioretinopathy and polypoidal choroidal vasculopathy.[43] Whether mast cell degranulation could be involved in such conditions should be explored. The fact that mast cells progressively reconstitute their granules evokes that if the stimulus persists, successive degranulations could lead to chronic barriers breakdown and sub-retinal fluid accumulation. In AMD, complement activation leads to C5a accumulation in the choroid, which could also induce mast cells degranulation[21] contributing to exudative complications.

In conclusion, since factors that trigger or result from mast cell degranulation have been identified in the pathogenesis of many "a priori" non-inflammatory diseases, including AMD, we suggest that mast cell degranulation could be an important factor in chronic ocular diseases as well as in acute ocular inflammation. Pharmacological inhibition of mast cell degranulation by inhibiting the release of inflammatory mediators could have an important therapeutic potential on retinal diseases associated with choroidal enlargement and/or serous retinal detachments.

Acknowledgements: The Fondation pour la Recherche Medicale is acknowledged for supporting Elodie Bousquet. This work has been supported by INSERM funds.

References

1. McMenamin PG, Polla E: Mast cells are present in the choroid of the normal eye in most vertebrate classes. Vet Ophthalmol 2013, .

2. LARSEN G: The mast cells in the uveal tract of the eye and changes induced by hormones and avitaminosis-C. Am J Ophthalmol 1959, 47:509–519.

3. Steptoe RJ, McMenamin C, McMenamin PG: Choroidal mast cell dynamics during experimental autoimmune uveoretinitis in rat strains of differing susceptibility. Ocul Immunol Inflamm 1994, 2:7–22.

4. De Kozak Y, Sainte-Laudy J, Benveniste J, Faure JP: Evidence for immediate hypersensitivity phenomena in experimental autoimmune uveoretinitis. Eur J Immunol 1981, 11:612–617.

5. LEVENE RZ: Mast cells and amines in normal ocular tissues. Invest Ophthalmol 1962, 1:531–543.

6. Galli SJ, Grimbaldeston M, Tsai M: Immunomodulatory mast cells: negative, as well as positive, regulators of immunity. Nat Rev Immunol 2008, 8:478–486.

7. Marshall JS: Mast-cell responses to pathogens. Nat Rev Immunol 2004, 4:787–799.

8. Enoksson M, Lyberg K, Möller-Westerberg C, Fallon PG, Nilsson G, Lunderius-Andersson C: Mast cells as sensors of cell injury through IL-33 recognition. J Immunol Baltim Md 1950 2011, 186:2523–2528.

9. Wershil BK, Wang ZS, Gordon JR, Galli SJ: Recruitment of neutrophils during IgE-dependent cutaneous late phase reactions in the mouse is mast cell-dependent. Partial inhibition of the reaction with antiserum against tumor necrosis factor-alpha. J Clin Invest 1991, 87:446–453.

10. Caspi RR: A look at autoimmunity and inflammation in the eye. J Clin Invest 2010, 120:3073–3083.

11. Wildner G, Diedrichs-Möhring M: Autoimmune uveitis and antigenic mimicry of environmental antigens. Autoimmun Rev 2004, 3:383–387.

12. De Kozak Y, Sakai J, Sainte-Laudy J, Faure JP, Benveniste J: Pharmacological modulation of IgE-dependent mast cell degranulation in experimental autoimmune uveoretinitis. Jpn J Ophthalmol 1983, 27:598–608.

13. Li Q, Whitcup SM, Fujino Y, Nussenblatt RB, Chan CC: The role of mast cells in endotoxin-induced uveitis. Invest Ophthalmol Vis Sci 1993, 34:256–259.

14. Da Silva PS, Girol AP, Oliani SM: Mast cells modulate the inflammatory process in endotoxin-induced uveitis. Mol Vis 2011, 17:1310–1319.

15. Nakai K, Gomi F, Ikuno Y, Yasuno Y, Nouchi T, Ohguro N, Nishida K: Choroidal observations in Vogt-Koyanagi-Harada disease using high-penetration optical coherence tomography. Graefes Arch Clin Exp Ophthalmol Albrecht Von Graefes Arch Für Klin Exp Ophthalmol 2012, 250:1089–1095.

16. MARMOR M: On the cause of serous detachments and acute central serous chorioretinopathy. Br J Ophthalmol 1997, 81:812–813.

17. Takeda A, Baffi JZ, Kleinman ME, Cho WG, Nozaki M, Yamada K, Kaneko H, Albuquerque RJC, Dridi S, Saito K, Raisler BJ, Budd SJ, Geisen P, Munitz A, Ambati BK, Green MG, Ishibashi T, Wright JD, Humbles AA, Gerard CJ, Ogura Y, Pan Y, Smith JR, Grisanti S, Hartnett ME, Rothenberg ME, Ambati J: CCR3 is a target for age-related macular degeneration diagnosis and therapy. Nature 2009, 460:225–230.

18. Tuo J, Grob S, Zhang K, Chan C-C: Genetics of immunological and inflammatory components in age-related macular degeneration. Ocul Immunol Inflamm 2012, 20:27–36.

19. Anderson DH, Radeke MJ, Gallo NB, Chapin EA, Johnson PT, Curletti CR, Hancox LS, Hu J, Ebright JN, Malek G, Hauser MA, Rickman CB, Bok D, Hageman GS, Johnson LV: The Pivotal Role of the Complement System in Aging and Age-related Macular Degeneration: Hypothesis Re-visited. Prog Retin Eye Res 2010, 29:95–112.

20. Schäfer B, Piliponsky AM, Oka T, Song CH, Gerard NP, Gerard C, Tsai M, Kalesnikoff J, Galli SJ: Mast cell anaphylatoxin receptor expression can enhance IgE-dependent skin inflammation in mice. J Allergy Clin Immunol 2013, 131:541–548.e9.

21. Nozaki M, Raisler BJ, Sakurai E, Sarma JV, Barnum SR, Lambris JD, Chen Y, Zhang K, Ambati BK, Baffi JZ, Ambati J: Drusen complement components C3a and C5a promote choroidal neovascularization. Proc Natl Acad Sci U S A 2006, 103:2328–2333.

22. Fischer MD, Huber G, Beck SC, Tanimoto N, Muehlfriedel R, Fahl E, Grimm C, Wenzel A, Remé CE, van de Pavert SA, Wijnholds J, Pacal M, Bremner R, Seeliger MW: Noninvasive, in vivo assessment of mouse retinal structure using optical coherence tomography. PloS One 2009, 4:e7507.

23. Spaide RF, Koizumi H, Pozzoni MC, Pozonni MC: Enhanced depth imaging spectral-domain optical coherence tomography. Am J Ophthalmol 2008, 146:496–500.

24. Omri S, Behar-Cohen F, de Kozak Y, Sennlaub F, Verissimo LM, Jonet L, Savoldelli M, Omri B, Crisanti P: Microglia/macrophages migrate through retinal epithelium barrier by a transcellular route in diabetic retinopathy: role of PKCζ in the Goto Kakizaki rat model. Am J Pathol 2011, 179:942–953.

25. Zhao M, Célérier I, Bousquet E, Jeanny J-C, Jonet L, Savoldelli M, Offret O, Curan A, Farman N, Jaisser F, Behar-Cohen F: Mineralocorticoid receptor is involved in rat and human ocular chorioretinopathy. J Clin Invest [Internet] 2012 [cited 2012 Jun 21], . Available from: http://www.ncbi.nlm.nih.gov/pubmed/22684104

26. Kim M, Kim H, Kwon HJ, Kim SS, Koh HJ, Lee SC: Choroidal thickness in Behcet's uveitis: an enhanced depth imaging-optical coherence tomography and its association with angiographic changes. Invest Ophthalmol Vis Sci 2013, 54:6033–6039.

27. Candia OA: Electrolyte and fluid transport across corneal, conjunctival and lens epithelia. Exp Eye Res 2004, 78:527–535.

28. Srinivas SP, Satpathy M, Guo Y, Anandan V: Histamine-induced phosphorylation of the regulatory light chain of myosin II disrupts the barrier integrity of corneal endothelial cells. Invest Ophthalmol Vis Sci 2006, 47:4011–4018.

29. Gardner TW, Lesher T, Khin S, Vu C, Barber AJ, Brennan WA Jr: Histamine reduces ZO-1 tight-junction protein expression in cultured retinal microvascular endothelial cells. Biochem J 1996, 320 (Pt 3):717–721.

30. Zawinka C, Resch H, Schmetterer L, Dorner GT, Garhofer G: Intravenously administered histamine increases choroidal but not retinal blood flow. Invest Ophthalmol Vis Sci 2004, 45:2337–2341.

31. Abraham SN, St John AL: Mast cell-orchestrated immunity to pathogens. Nat Rev Immunol 2010, 10:440–452.

32. Marshall JS, Jawdat DM: Mast cells in innate immunity. J Allergy Clin Immunol 2004, 114:21–27.

33. De Vos AF, van Haren MA, Verhagen C, Hoekzema R, Kijlstra A: Kinetics of intraocular tumor necrosis factor and interleukin-6 in endotoxin-induced uveitis in the rat. Invest Ophthalmol Vis Sci 1994, 35:1100–1106.

34. Zech JC, Pouvreau I, Cotinet A, Goureau O, Le Varlet B, de Kozak Y: Effect of cytokines and nitric oxide on tight junctions in cultured rat retinal pigment epithelium. Invest Ophthalmol Vis Sci 1998, 39:1600–1608.

35. Malaviya R, Ikeda T, Ross E, Abraham SN: Mast cell modulation of neutrophil influx and bacterial clearance at sites of infection through TNF-alpha. Nature 1996, 381:77–80.

36. Hoekzema R, Verhagen C, van Haren M, Kijlstra A: Endotoxin-induced uveitis in the rat. The significance of intraocular interleukin-6. Invest Ophthalmol Vis Sci 1992, 33:532–539.

37. Leung BP, Culshaw S, Gracie JA, Hunter D, Canetti CA, Campbell C, Cunha F, Liew FY, McInnes IB: A role for IL-18 in neutrophil activation. J Immunol Baltim Md 1950 2001, 167:2879–2886.

38. Robertson SE, Young JD, Kitson S, Pitt A, Evans J, Roes J, Karaoglu D, Santora L, Ghayur T, Liew FY, Gracie JA, McInnes IB: Expression and alternative processing of IL-18 in human neutrophils. Eur J Immunol 2006, 36:722–731.

39. Rosenbaum JT: Eyeing macular degeneration--few inflammatory remarks. N Engl J Med 2012, 367:768–770.

40. Shen J, Choy DF, Yoshida T, Iwase T, Hafiz G, Xie B, Hackett SF, Arron JR, Campochiaro PA: Interleukin-18 has Antipermeablity and Antiangiogenic Activities in the Eye; Reciprocal Suppression with VEGF. J Cell Physiol 2014, .

41. Doyle SL, Campbell M, Ozaki E, Salomon RG, Mori A, Kenna PF, Farrar GJ, Kiang A-S, Humphries MM, Lavelle EC, O'Neill LAJ, Hollyfield JG, Humphries P: NLRP3 has a protective role in age-related macular degeneration through the induction of IL-18 by drusen components. Nat Med 2012, 18:791–798.

42. Tarallo V, Hirano Y, Gelfand BD, Dridi S, Kerur N, Kim Y, Cho WG, Kaneko H, Fowler BJ, Bogdanovich S, Albuquerque RJC, Hauswirth WW, Chiodo VA, Kugel JF, Goodrich JA, Ponicsan SL, Chaudhuri G, Murphy MP, Dunaief JL, Ambati BK, Ogura Y, Yoo JW, Lee D, Provost P, Hinton DR, Núñez G, Baffi JZ, Kleinman ME, Ambati J: DICER1 loss and Alu RNA induce age-related macular degeneration via the NLRP3 inflammasome and MyD88. Cell 2012, 149:847–859.

43. Warrow DJ, Hoang QV, Freund KB: PACHYCHOROID PIGMENT EPITHELIOPATHY. Retina Phila Pa 2013, .

44. Protein Kinase C_ (PKC_) Regulates OcularInflammation and Apoptosis in Endotoxin-Induced Uveitis (EIU) Signaling Molecules Involved in EIU Resolution by PKC_ In*hibitor and Interleukin-13. The American Journal of Pathology, Vol. 170, No. 4, April 2007*.

Figure legends

Figure 1. Clinical examination and grading of the pathological signs in the anterior segment of the eye after subconjunctival injection of 48/80.

A. Slit lamp photographs were obtained from rats injected in the conjunctiva with 48/80. The eyes were observed from 10 min up to 7 hrs after injection. Edema of the conjunctiva was detected from 10 min after 48/80 injection (arrow). A cataract was observed in 17 out of 25 eyes (68%), from 1 hr (asterisk) to diminish progressively up to 7 hrs. At 7 hrs in this eye, haemorrhages were observed in the iris (arrow) and in the conjunctiva (arrowhead).

Original magnification: X 7; C : conjunctiva; i : iris ; co : cornea;

n = 7 rats injected with 48/80

n = 25 rats for observation of cataract in the right eyes

B. *Graph showing the clinical grading of rats injected with 48/80 in the conjunctiva of right eyes and non-injected in the controlateral left eyes and observed at the slit lamp from 15-30 min to 24 hrs. Clinical signs developed from 15-30 min and peaked at 1hr-2hrs, to decrease from 3hrs. The intensity of clinical signs, compared to pic levels found at 1-2 hrs, significantly decreased at 3, 4, 5, 7 hrs and 24 hrs. Non-injected left eyes did not show any clinical abnormalities. n = 6 rats injected with 48/80 in the right eyes and not injected in left eyes*

**P<0.5; *** P<0.001*,

Figure 2

A-H: Morphological alterations of mast cells in flat mounts of the choroid at different time points after subconjunctival injection of 48/80.

At all time intervals in the left non-injected eye (A-C and insert), numerous normal mast cells were detected along choroidal vessels. In contrast, in the right 48/80-injected eyes, mast degranulation was visible at all time intervals tested as mast cell granules dispersed in the surrounding tissue (D-F). The intensity and number of degranulated mast cells increased with time, detectable in numerous mast cells at 1 hr (D) (insert) and to be maximum at 3 hrs (E) (insert). Extensively degranulated cells were visible with exocytosis of granules (D-E). At 24 hrs (F), all mast cells presented an abnormal shape with dispersed granules intensively stained in blue with toluidine blue.

Photomicrographs were obtained from one eye/time point and are representative of similar flat mounts stained from 3 rats/time point.

Scale bar: 30 μm, insert X3

G, H: Counting of choroidal mast cells

G. The number of degranulated mast cells was significantly increased at 1 hr and 3 hrs in 48/80-injected eyes compared to non-injected eyes. Low number of degranulated mast cells was detected at all time points in non-injected eyes.

H. Correlatively, a significant decrease of the number of non-degranulated mast cells in 48/80-injected eyes was detected at 1 hr and 3 hrs compared to non-injected eyes. High number of non-degranulated mast cells was detected at all time points in non-injected eyes.

Data are means ± SEM, n = 3 rats per condition, * $p<0.05$.

Figure 3: Pathological effects of mast cell degranulation in ocular tissues.

Historesin sections of left non-injected eyes and right 48/80-injected eyes between 1 hr and 3 hrs-24 hrs after 48/80 injection (A-H).

In the left non-injected eyes (A, C) normal mast cells (inserts) were observed in the vicinity of vessels at the limbus of the cornea (A) and near choroidal vessels (C). Normal aspect of the ocular structures: cornea with endothelial cells regularly distributed (asterisk) (B), normal retina (C) and choriocapillaris (C, asterisk).

In contrast, in the right 48/80-injected eyes (D-H), at 1-3 hrs, degranulated mast cells were observed in the vicinity of ocular vessels at the limbus of the cornea (D, insert), in the conjunctiva (E). From 1-3 hrs to persist up to 24 hrs after 48/80 injection, an important infiltration of PMNs (F, insert, arrowhead) and macrophages (F, insert, arrow) was observed in the aqueous humor behind the vacuolated endothelium of the cornea (F, asterisk). Important dilation of the choriocapillaris was observed (G, asterisk) associated with focal retinal detachment (G). A mobilized RPE cell was present in the subretinal space (G, arrowhead). Starting at 1 hr (G) after 48/80 injection to be very important at 3 hrs-24 hrs (H), alterations of the retinal nuclear cell layers with picnotic aspects of the nuclei visible at the level of the retinal detachment (H, asterisk). Fibrin deposits were seen all along the RPE (G, arrow). At 24 hrs, large retinal tissue destruction was observed (H).

Retinal sections were immunostained by TUNEL assay (green) and DAPI (blue) (I-K). Normal retina was observed in the left non-injected eye (I). In contrast apoptotic retinal cells (TUNEL-positive nuclei) were detected in the three nuclear cell layers of the retina at 3 hrs

after 48/80 injection (J) and at 24 hrs (K) that will lead to the extensive lesions of retinal cells shown in historesin section (H, asterisk).

48/80-injected eyes: n= 3; contralateral non-injected eyes: n=3.

Original magnification: scale bar: 30 μm (insert X4)

Figure 4. Serous retinal detachment and choroidal vessels dilation after subconjunctival injection of 48/80.

Optical Coherence Tomography (OCT) (A, B, D, E, G, H) and corresponding historesin sections (C, F, I) at different time points after 48/80 injection.

At 1 hr, undulations and hyperreflective areas were detected at the level of retinal pigmented epithelium (A, B, arrows) corresponding to small retinal detachments on histological sections (C, arrows). From 3 hrs to 24 hrs after injection of 48/80, retinal serous detachments were observed with presence of subretinal fluid underneath (D, E, F, G, stars). Edematous areas were detected in the retina (C, F, arrowheads). An important dilation was detected in the choriocapillaries (star) and choroidal vessels (asterisk) (I).

At 1 hr: n = 6, 3-6 hrs: n = 6

Scale bar: 25 μm

GCL: ganglion cell layer; IPL: inner plexiform layer; INL: inner nuclear layer; OPL: outer plexiform layer; ONL: outer nuclear layer; RPE: retinal pigmented epithelium; Ch: choroid.

Figure 5. Effect of subconjunctival injection of 48/80 on choroidal thickness.

Choroidal thickness was measured on photographs taken on historesin sections from the optic nerve to the ciliary body.

A, B. At 1 hr and 3 hrs after injection of 48/80, choroids from eyes injected with 48/80 (n = 6) showed a localized significant increase of thickness compared to contralateral eyes (n = 6) and NaCl-injected eyes (n =5). **C, D**. At 6 hrs and 24 hrs, a significant and extended increase of choroidal thickness was shown in choroids from rats injected with 48/80 (n = 6) compared to contralateral eyes (n = 6) and NaCL-injected eyes (n = 6).

Figure 6. Effects of mast degranulation on RPE cell jonctions by subconjunctival injection of 48/80.

A-D: RPE flat mounts immunostained with occludin (green) and DAPI (blue) after injection of 48/80 or NaCl. In NaCl controls, occludin labeled the regular hexagonal membrane of RPE cells (A), whereas 1 hr after injection of 48/80, enlarged RPE cells were seen (B, C, stars)

with irrregular occludin labeling originating from loss of junctions between some RPE cells (D, arrowhead). 48/80-injected eyes: n = 6; NaCl-injected eyes: n = 5

E-G: On corresponding historesin sections, in controls, RPE cells were regularly distributed between the photoreceptor cells and the choroid (E, arrows). In contrast, after injection of 48/80, RPE cells were altered or missing (F, asterisk). In some wide places, RPE cells were separated from one to another, showed a round shape and were detached from the basal membrane (G, white arrows) in front of a retinal detachment area.

Photomicrographs are representative of similar sections stained from 3 rats per each time point. Scale bar: 25 μm

Figure 7. Time course of intra-ocular cytokine and chemokine concentrations after subconjunctival injection of 48/80.

TNF-α was increased at 1 hr and 3 hrs after 48/80 injection as compared to NaCl-injected eyes (A). A significant upregulation of IL-5 at 3 hrs, 6 hrs and 24 hrs was found in 48/80-injected eye (B), together with an upregulation of IL-6 and CCL-2 at 6 hrs and 24 hrs (C, D) as compared to NaCl-injected-eyes. An increase of IL-1β and CXCL-1 (E, F) was detected at 24 hrs. A significant increase of IL-18 (G) and a significant decrease of VEGF (I) occurred at 24 hrs in 48/80-injected eyes. The course of levels of IL-18 (H) and VEGF (J) in 48/80-injected eyes was opposite. At 3 hrs, IL-18 significantly decreased compared to its levels at 1 hr, whereas VEGF significantly increased compared to its levels at 1 hr. In contrast compared to levels at 3 hrs, at 6 hrs and 24 hrs IL-18 significantly increased whereas VEGF significantly decreased.

48/80-injected eyes: n = 4-6 per time point; NaCl-injected eyes (control) : n = 5-6 per time point