Mast cells profiles and heterogeneity in lipopolysaccharide-induced uveitis model

Alves JP 1, da Silva VF 1, Tencarte SR 1, Montresor LB 1, Pereira GH 1, Possebon L 1, 2, Souza HR 1, 2 and Girol AP 1, 2, *

1 University Center Padre Albino (UNIFIPA), Catanduva, SP, Brazil.
2 São Paulo State University, (UNESP), Institute of Biosciences, Humanities and Exact Sciences (IBILCE), São José do Rio Preto Campus, SP, Brazil.

Publication history: Received on 12 November 2019; revised on 25 November 2019; accepted on 28 November 2019

Article DOI: https://doi.org/10.30574/wjarr.2019.4.2.0089

Abstract

Background: Uveitis is an important inflammation of the middle tunica of the eye which can lead to blindness. Mast cells (MCs) participate in the development of the inflammatory process by attracting other cell types. Methods: The MCs profile was evaluated in the endotoxin-induced uveitis model (EIU), in the acute (24 hours) and late (48 hours) phases of the inflammatory process. EIU was induced by inoculation of lipopolysaccharide, subcutaneously, into the paw of the rats. After 24 and 48 hours of the induction, the eyes were enucleated for paraffin inclusion and histopathological studies of MCs in the anterior chamber of the eye. MCs were morphologically evaluated using Toluidine blue dye, their histamine accumulation was analyzed by Alcian blue and Safranin-O method, and the presence of tryptase and chymase by immunohistochemistry. Results: The results showed larger amount of total and degranulated MCs, as well as Alcian blue and Safranin positive MCs in the acute phase of EIU, especially in the ciliary body. Few tryptase positive MCs were observed in acute and late phases of EIU, but numerous chymase positive MCs were found in the 24-hour group. Conclusions: The data highlight the MC modulation during EIU and indicate these cells closely linked to the inflammatory process in uveitis.

Keywords: Inflammatory cells; Mast cell; Ocular inflammation; Histamine

List of Abbreviations

ANOVA = Analysis of variance, AqH = Aqueous humor, ARVO = Association for Research in Vision and Ophthalmology, BSA = Bovine serum albumin, DAB = Diaminobenzidine, EIU = Endotoxin induced uveitis, IL-1β = Interleukin 1 beta, LPS = Lipopolysaccharide, MCs = Mast cells, MMPs = Matrix metalloproteinases, PBS = Buffered saline, S.E.M = Standard error mean, TGF β1 = Transforming growth factor beta 1, TLR4 = Toll-like receptors 4, TNF-α = Tumor necrosis factor alpha

1. Introduction

Uveitis is an ocular inflammation characterized by leukocyte accumulation, and is an important cause of blindness [1,2]. This ocular abnormality can be caused by immune-mediated processes or infectious organisms and especially affects the uvea, composed of the iris, ciliary body and choroid [3]. Currently, uveitis is treated with corticosteroids, chemotherapeutics, and tumor necrosis factor-alpha (TNF-α) inhibitors; however, side effects such as increased ocular pressure and cytotoxicity limit the use of these drugs [4].

The endotoxin induced uveitis (EIU) [5] is a widely used model that allows the understanding of this ocular inflammation [6-8]. Lipopolysaccharide (LPS) is used as an inducer of EIU and binds to toll-like receptors 4 (TLR4)
stimulating the production and release of pro-inflammatory mediators [9]. The increase of the inflammatory mediators promotes the breakdown of the blood-ocular barrier, leading to the infiltration of inflammatory cells into aqueous humor (AqH) and extravasation of proteins [7,10]. In experimental uveitis, the neutrophil migration in the iris occurs from two to four hours after endotoxin administration [11]. The inflammatory process of uveitis peaks at 24 hours after LPS injection (acute phase of inflammation) and gradually regresses within the next 24 hours (late phase of inflammation) [6, 7, 12].

Most of the chemical mediators available in inflammatory processes come from mast cells (MCs) [13,14]. The content of the MCs granules can be released by the activation of IgE to FcεR receptors, but also by the binding of TLR receptors by microbial products [13,14]. These specialized secretory cells arise from the bone marrow and are distributed in the connective tissues of the body through the circulation [14,15,16]. MCs complete their differentiation after tissue infiltration, developing special properties related to the content of their granules, that are rich in histamine and specific proteases [15,16]. In murines, the maturation of MCs can be analyzed according to the accumulated amount of histamine, which can be evidenced by the reaction of histamine to Safranin-O dye [17-19].

MCs present important functions in the acute phase of inflammation because in their degranulation process, besides the release of chemotactic factors, they also secrete proteases such as tryptase and chymase into the extracellular medium, contributing to tissue degradation and angiogenesis [15, 20]. Both proteases cleave terminal fractions of proteins to make them active. Tryptases cleave proteins in trypsin and serine residues and chymases in serine residues. Moreover tryptase activates urokinase and matrix metalloproteinases (MMPs) while chymase activates, in addition to MMPs, interleukin (IL)-1β, transforming growth factor (TGF)-β1 and converts angiotensin I to II [14,15,20,21].

Although there are few studies of MCs in uveitis, investigations have indicated the relevance of these cells for modulation of this ocular inflammation [6]. For this reason, we investigated in vivo the profiles of MCs in the EIU, related to activation, maturation and heterogeneity, enhancing previous studies to better understand the biology of these cells in ocular inflammatory processes, especially in uveitis.

2. Material and methods

2.1. EIU induction protocol

Male Wistar rats, 6 to 8 weeks of age (150 to 200 g) were kept in cages in a temperature controlled environment (23 to 25 °C) and received water and food ad libitum. The experimental procedures were conducted in compliance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research, also according to the guidelines for biomedical research stated by the Brazilian Societies of Experimental Biology and the European legislation on care and use of experimental animals (EU Directive 2010/63/EU for animal experiments; R.D. 53/2013) and after approval of the Ethics Committee for the Use of Animals of University Center Padre Albino, Catanduva, SP, Brazil (No. 11/14). The experiments were designed to minimize the number of animals used and their suffering during the execution of the protocols and are in accordance with other works developed by our research group [19,22]. To perform the histopathological, immunohistochemical and other set of experiments, the number of animals has been evaluated by PS Power© 3.0. All animals were daily examined by the institution’s veterinarian.

For the development of EIU, rats were anesthetized with isoflurane (1%) and inoculated subcutaneously in the right footpad with 200 μg (1mg/kg) of LPS (Escherichia coli serotype 0127: B8, Sigma Chemical Co. Poole, Dorset, UK) diluted in 100 μl of buffered saline (PBS) [6-8]. The animals were divided into two experimental groups (n = 6/group): EIU 24 hours (acute phase of inflammation) and 48 hours (late phase of inflammation). Animals without manipulation were used as controls. After the induction periods, the rats were euthanized by excessive doses of anesthetic.

2.2. Histopathological and immunohistochemical analyses

The eyes were collected, fixed in 4% formalin, and processed for inclusion in paraffin. Sections of 3 μm were used for histopathological and immunohistochemical studies. The MCs were evaluated according to their morphological characteristics in intact and degranulated, by Toluidine Blue 0.1%. Alcian Blue dye was used to evidence MCs with low amounts of histamine and Safranin-O 2.5% staining was performed to evidence MCs with increased amounts of histamine [19].

Immunohistochemical reactions were developed to verify the presence of tryptase and chymase proteases in MCs granules. Sections of different samples were prepared on gelatinized slides and then deparaffinized and rehydrated.
After antigen retrieval (citrate buffer pH 6.0 at 96 °C for 20 minutes) and the blocking of endogenous peroxidase, the sections were washed in PBS and incubated with the primary monoclonal mouse antibodies: anti-tryptase (1: 200; Millipore Corporation: catalog nº MAB1222; Darmstadt, Hessen, Germany) and anti-chymase (1:40; Abcam: catalog nº ab186417, Cambridge, Cambridgeshire, UK) diluted in 1% bovine serum albumin (BSA). The next day, they were incubated with the biotinylated secondary antibody (Zymed Invitrogen kit: catalog nº 95-9943, Camarillo, California, USA), and then on a diaminobenzidine (DAB) substrate (Zymed Invitrogen kit: catalog nº 750118, Pittsburgh, Pennsylvania, USA) for development. Afterwards, the sections were counterstained with hematoxylin. Negative control of the reaction was obtained by omission of the primary antibody from the process described above.

### 2.3. Statistical analyses

In all preparations and groups the MCs were quantified in the anterior chamber of the eye, mainly in the region of the ciliary body. The quantification of MCs was performed in 5 different images per slide (3 slides/group), obtained by the objective of 40X in the Leica DM50 microscope and selected subjectively scattered (representative fields scattered in the preparation, observer random mode). The areas were obtained with the Leica Image Analysis software. Data were expressed as mean ± standard error mean (S.E.M.) of the number of MCs per mm².

The results were previously submitted to the descriptive analysis and normality determination (by GraphPad Prism® 6.01 software, São Paulo-SP, Brazil) and, as the samples presented normal distribution, the Analysis of Variance (ANOVA) was used, followed by Bonferroni test. All values were expressed as mean ± S.E.M. and P values less than 0.05 were considered statistically significant.

### 3. Results

LPS administration promoted intense leukocyte influx, especially neutrophils, after 24 hours of inoculation, characterizing the acute phase of inflammation (Figure 1C and D). The influx of inflammatory cells occurred mainly in the anterior region of the eye. In the animals after 48 hours, considered the late phase of inflammation, there was reduction of inflammatory cells (Figure 1E and F) and in the control animals no extravasated leukocytes were observed (Figure 1A and B).

After toluidine blue staining, we observed the MCs in the iris and ciliary body (Figure 2A, B and C). Our results showed higher number of total MCs, identified by the presence of metachromatic cytoplasmic granules in the 24 hours EIU group (47.91 ± 7.85 p < 0.05 vs Control: 14.58 ± 5.96; Figure 2F), most of them were observed in degranulation state (Figure 1E and G), with the granules scattered around the cell. In contrast, most MCs found in the control and 48 hours EIU groups showed intact cell characteristics with well-defined contours (Figure 2D and G).

Regarding the analyses of the presence of histamine, a greater number of positive Alcian blue MCs (54.1%) was observed in the acute inflammation group (Figure 3A). Again, in the 24 hours EIU group most of the MCs were safranin positive (Control: 1.25 ± 1.02 p < 0.001 vs 24h EIU: 23.95 ± 5.45 p < 0.01 vs 48h EIU: 6.25 ± 2.28; Figure 3B) indicating greater amount of histamine in MCs of the acute phase of inflammation.

Immunohistochemical studies revealed the presence of both proteases in the MCs of all evaluated groups. Interestingly, few tryptase positive cells were observed in the 24 hours (25%) and 48 hours (16.7%) EIU groups (Figure 3C) compared to control (58.3%). However, numerous chymase positive MCs were observed in the 24 hours EIU group (42.8%) (Figure 3D) compared to the others (Control: 37.5% and 48h: 21.4%).
Figure 1 Histopathological analyses of the anterior ocular segment. Absence of inflammatory cells in the anterior segment of the eye (A and B) and intense leukocyte influx (arrows) after 24 hours of administration of LPS, acute phase of inflammation (C and D). Few cells after 48 hours of EIU induction (E and F). n = 6/group. Hematoxilin and Eosin stain. Bars: A, C and E, 200 μm; B, D and F, 50 μm.

Figure 2 MCs activation in EIU: Few MCs in control (A) and EIU 48h (C) groups. Increased amount MCs in EIU 24h (C). Intact MC (D). MC in degranulation process (E). n = 6/group. Toluidine blue stain. Bars: 50 μm. Total (F) and degranulated (G) MCs quantification.
Figure 3. Heterogeneity of MCs in EIU: MCs with low accumulation of histamine, Alcian blue positives (A) and with accumulation of histamine, Safranin positives at EIU 24h (B). MCs immunomarked for tryptase (C) and chymase (D). n = 6/group.

4. Discussion

MCs are essential in the fight against bacterial infections because they mobilize neutrophils to the site of infection [23]. Investigations show that the degranulation of MCs contribute to inflammation in EIU [6]. However, there is little research demonstrating the profile of MCs in the EIU and, consequently, a better understanding of the role of these cells in uveitis. Therefore, we evaluated the degranulation stage, the accumulation of histamine and the presence of tryptase and chymase in the MCs present in the anterior segment of the eye in LPS-induced uveitis.

The first analysis of the MCs, through the staining with Toluidine blue, showed larger numbers and higher degranulation of these inflammatory cells after 24 hours of EIU induction, period of greater influx of neutrophils into aqueous humor. In a previous study, numerous MCs were also observed in the acute phase of inflammation [6], especially in the anterior segment of the eye and coincident with the influx of leukocytes after the rupture of the blood-ocular barrier. Thus, our results reinforce the action of MCs in the acute phase of the inflammatory process, as these cells release chemotactic and vasodilatory factors that modulate inflammation [20,23-25].

We found a reduction in the number of MCs after 48 hours of LPS inoculation, both intact and degranulated, compared to the acute phase of inflammation. However, most MCs observed in the late phase were intact. Again, these data are in agreement with the study by Silva and colleagues [6] and suggest that MCs can resynthesize their cytoplasmic granules after initial stimulation.

In order to verify the resynthesis of the cytoplasmic granules of MCs in the late stage of the EIU, we performed studies of maturation of these cells, using the Alcian blue and Safranin-O stains [19]. Our studies revealed large numbers of MCs both Alcian blue and Safranin positive in the acute phase of inflammation. Interestingly, there was no significant difference related to positive Alcian blue MCs between both groups of inflammation, 24 and 48 hours, indicating that many immature MCs are present throughout the inflammatory process, which suggests resynthesis. In the late phase of the inflammatory process, a few MCs were Safranin positive, indicating that there is accumulation of histamine in these cells and that this mediator is released in large quantities in the acute phase, where most of the MCs are in a degranulation state.

MCs also produce other potent biologically active mediators, as tryptase and chymase, which contribute to the promotion of angiogenesis [26,27]. For these reasons, we verified the heterogeneity of MCs by quantification of tryptase and chymase in the acute and late phases of EIU. Our analyses indicated the presence of both proteases, but tryptase was found in greater quantity in the control animals. Most chymase positive cells were especially found in the acute phase of the inflammatory process. The small amount of tryptase in MCs during EIU may be related to the degranulation process of these cells, with tryptase being related to leukocyte influx and angiogenesis [14].
Curiously, the quantification of the positive safranin MCs and the observed heterogeneity of these cells indicated a link between the histamine accumulation of MCs and the accumulation of chymase, reinforcing the process of resynthesis and maturation. The study by Nakazawa and colleagues [18] demonstrated the importance of histamine for the maturation of MCs in which histidine decarboxylase deficient mice showed decreased chymase expression in MCs. In addition, another study indicated that chymase is the first protease to be expressed in large amounts by MCs [28]. Thus, our data indicate the involvement of tryptase and mainly chymase in LPS-induced uveitis.

5. Conclusion
The data obtained point that MCs alter their morphological, histochemical and heterogeneity profiles during the inflammatory process of EIU. In the acute phase of inflammation, MCs degranulate releasing chemical mediators, contributing to the severity of the inflammatory process. In this phase, the main protease is chymase, related to the increase of histamine and important to the exacerbation of the inflammatory process. Hence, our data reinforce the modulatory role of MCs in the development of uveitis.

Compliance with ethical standards

Acknowledgments
This research was supported by Grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq (404190/2016-2) and University Center Padre Albino UNIFIPA.

Disclosure of conflict of interest
There is no conflict of interest.

Statement of ethical approval
The experimental procedures were conducted in compliance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research, also according to the guidelines for biomedical research stated by the Brazilian Societies of Experimental Biology and the European legislation on care and use of experimental animals (EU Directive 2010/63/EU for animal experiments; R.D. 53/2013) and after approval of the Ethics Committee for the Use of Animals of University Center Padre Albino, Catanduva, SP, Brazil (No. 11/14).

References


How to cite this article