Research Article

The cellular inflammatory response associated with sulfur mustard induced delayed limbal stem cell deficiency and evaluation of tacrolimus treatment

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Abstract

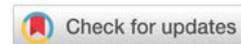**Purpose:** To further investigate the inflammatory response as one of the leading factors in the development of delayed limbal stem cell deficiency, clinically manifested by corneal neovascularization , following sulfur mustard ocular exposure in rabbits and to evaluate potential therapy.

Materials and methods: Right eyes of rabbits were exposed to sulfur mustard vapor. Clinical examination was performed and the growth of corneal blood vessels was evaluated. Inflammatory cells (neutrophils, macrophages, antigen-presenting cells and T cells) were identified immunohistochemically. Tacrolimus (Prograf, Teva, 5mg/ml) an immuno-modulator drug, was tested via sub-conjunctival injection (50µl), started either before or after the development of neovascularization (72h and 4weeks, respectively) given once a week for 3weeks.

Results: Limbal stem cell deficiency, associated with inflammatory-related corneal neovascularization was developed starting at two weeks post-exposure. The cellular inflammation included elevation of neutrophils in the limbus at one week and later (2-4weeks) in the cornea. Antigen-presenting cells (predominantly dendritic cells) were seen in the limbus and the cornea at 1-4w, together with a massive accumulation of T cells that were correlated with neovascularization score. Treatment with tacrolimus was not effective in reducing the clinical signs or inflammatory infiltration.

Conclusions: Sulfur mustard-induced delayed limbal stem cell deficiency associated with corneal angiogenesis and inflammatory infiltration of neutrophils, dendritic cells and T cells in the limbus and cornea was observed. In spite of the association between T cells infiltration and corneal neovascularization, treatment with tacrolimus, did not reduce the inflammatory response and was not beneficial as a single drug therapy against the late sulfur mustard ocular injury.

Introduction

Sulfur Mustard (SM) is a potent warfare agent, rapidly absorbed in the skin and mucous surfaces and is known by its ability to cause vesication, as well as prolonged injuries mainly to the eyes, skin and respiratory system. The eyes are the most sensitive organ to SM exposure and the lesions are related to the dose and exposure duration. Yet, since its first use in World

War I and despite vast efforts, only limited success has been attained to find an effective treatment to prevent or reduce these lesions.

Ocular injuries following exposure to SM are characterized by corneal erosions and acute inflammation of the anterior segment, that after a clinically silent period may deteriorate into late pathology in part of the exposed eyes. The later is expressed by corneal Neovascularization (NV), chronic inflammation and epithelial defects [1-8].

We have previously shown that the late pathology is derived from a gradual loss of corneal epithelial stem cells, residing in the limbus, leading to Limbal Stem Cell Deficiency (LSCD) disorder [3]. Furthermore, we have shown that the LSCD associated with SM ocular toxicity was not due to a direct cytotoxic effect of the agent on the epithelial stem cells, but rather from secondary events at the limbal niche, that produced a pathological microenvironment for the limbal epithelial stem cells, triggering their death and subsequently the delayed LSCD [9]. Indeed, a prolonged nerve impairment and a focal inflammatory infiltration were observed in the limbus of eyes that developed LSCD following SM exposure [10].The inflammatory response in eyes displaying the late ocular injury (clinically impaired eyes) included increased edema, higher levels of matrix metalloproteinases [11] and inflammatory mediators, predominantly MCP-1, IL-8, IL-6 and IL-β in the limbus [12]. This is consistent with the reports in the literature on the strong relationship between the maintenance and the impaired viability of stem cells with inflammation [13,14].

Supporting the role of inflammation in the pathogenesis of SM-induced delayed LSCD, are our previous results, indicating the beneficial effects of anti-inflammatory treatments [3,15]. We have shown that steroid treatment (dexamethasone) attenuated the incidence and the severity of the late clinical symptoms, when administered as a post-exposure preventive treatment during the acute phase, or as a symptomatic therapy during the late phase. Yet, the symptomatic steroid treatment was efficient only as long as the treatment sustained, and a long-term use of steroids is not recommended due to side effects.

Thus, further investigation of the inflammatory response associated with SM-induced delayed LSCD may contribute to the understanding of the role of inflammation in this pathogenesis and for improving current therapy.

The aim of the present study was to characterize the inflammatory infiltrating cell populations, associated with SM induced ocular injury and their temporal distribution focusing on the limbal stroma, where the stem cells niche is located [16].

In general, the infiltrating cells that are involved in ocular inflammation associated with NV, are initially neutrophils, followed by macrophages that sustain the angiogenic cascade by producing angiogenic factors and proteolytic enzymes [17] and by dendritic cells that are involved in the activation of pathogenic T cells [18]. Nevertheless, the involvement of these cells in SM induced ocular injuries is still obscure. In addition,

it is not clear if the angiogenic cascade described above is involved in the delayed LSCD following SM exposure. Thus, better understanding of the cellular inflammatory response in SM induced ocular injury may point out towards more specific therapeutic targets.

Following our current findings showing the involvement of T cells in limbal inflammation and neovascularization, we evaluated the therapeutic potential of the anti-inflammatory macrolide, tacrolimus (FK-509) which is known as a T cell suppressor [19,20]. Tacrolimus is a potent immunosuppressant that has been used in tissue transplantations to prevent allograft rejection [21] and in atopic dermatitis [22]. In addition, tacrolimus was shown to be efficient in immune and in inflammatory disorders of the ocular anterior segment as an anti-inflammatory drug that may replace or reduce the use of higher doses of steroids [19,23,24]. Its anti-inflammatory capacity affects various components of the inflammatory cascade including an anti-angiogenic effect [24,25]. In the present study, tacrolimus was evaluated as an early post-exposure preventive therapy, prior to significant T cell infiltration, before the development of LSCD, and as a symptomatic therapy after the clinical manifestation of LSCD and corneal NV, as a candidate to replace steroids.

The present study was carried out in our well-established SM-induced ocular injury model in rabbits, which mimics the injury in humans and serves to evaluate the beneficial effect of various treatments [11,15,26].

Material and methods

Animals

New Zealand White female rabbits (Charles River, Canada or Envigo Israel), weighing 2-3kg were used. Food and water were provided ad libitum and a 12h light/dark cycle was maintained. All procedures involving animals were in accordance with the NIH Guide for the Care and Use of Laboratory Animals. The protocols were approved by the Institutional Animal Care and Use Committee.

Experimental outline

Forty-eight animals were used. Details of the experimental model have been previously described [2,3]. Briefly, neat SM (10μl, over 95% purity) was applied to a filter paper disc held within glass goggles attached to the right eye for a period of four minutes, aiming to produce moderate to severe ocular lesions. A clinical follow-up was performed daily, during the first week following SM exposure, and then once a week up to four (characterization study) or seven weeks (treatment study), including slit-lamp examination and pachymetry (see details below).

Animals were euthanized by an overdose of sodium pentobarbitone (1-2ml, 200mg/ml) via ear vein injection, at various time intervals following exposure (0, 1, 2 and 4weeks) and 7weeks following treatment. Eyes were enucleated, fixed in 4% neutral buffered paraformaldehyde for histology or immunohistochemistry (see below).

Clinical observations

Slit-lamp biomicroscopy examinations of the eyes including fluorescein staining for corneal erosions were carried out as previously described [2,3]. The clinical findings were documented by photography and scored according to our semi-quantitative scoring scale [3]. The extent of NV was analyzed by measurement of corneal blood vessels' length in digital pictures, using computerized image analysis software (Image-Pro, Media Cybernetics).

Pachymetry

Peripheral and central corneal thickness were measured with an ultrasonic pachymeter (Pachette 3, DGH Technology, INC). Before pachymetry, eyes were locally anesthetized using 0.4% Benoxinate HCl (Localin ophthalmic solution, Fischer). Measurements were performed at the center of the cornea and in the superior and inferior limbal quadrants.

Histology

Following fixation, eyes were processed routinely for paraffin embedding. Four to six microns thick mid-sagittal (temporal to nasal) sections were cut serially to include conjunctiva, limbus and cornea. Selected sections were stained with hematoxylin and eosin (H&E) for general morphology.

Quantitative analysis of the number of cells was carried out on digital pictures taken from H&E stained sections from limbal regions at x60 objective magnification, utilizing a computerized image analysis system (Image-Pro Express 4.0). The number of cells in the limbus was counted in the sub-epithelial upper stroma in 9 different arbitrary areas in each eye (n=4/group).

Immunohistochemistry

Identification of the cellular infiltration was performed in free floating frozen sections as follows: Following fixation, eyes were immersed in 4% neutral buffered paraformaldehyde containing 30% sucrose overnight, until tissues sunk, and then eyes were frozen at -70°C until further processing. Frozen sections (50 μm thick) were cut serially at mid-sagital plane. Inflammatory cells were identified, using immunofluorescence staining with the following primary monoclonal antibodies: anti-NP5 monoclonal antibody (clone R3, Hycult, 1:500) for neutrophils, anti-MHC class II (clone 2C°-4, Serotec 1:500) for antigen-presenting cells (APCs), RAM11 anti-macrophage (Mouse Ab-3, Clone JH121, 1:100, 1:250, 1:500) for macrophages, and anti-CD43 (clone L11/135, Serotec, 1:500) for T cells. Fluorescence secondary antibodies (Alexa fluor-594 goat anti-mouse) served for labeling. The protocol included permeabilization in 0.2% Triton X-100 in PBS (2hrs), blocking in 10% normal goat serum in phosphate buffer saline (2-4 h), incubation with the primary antibodies for 48 h at 4°C and incubation with the secondary antibody for 48 h at 4°C, counterstaining with DAPI (0.3mg/ml for 5 min, Sigma) and mounting with Fluoromount. For control sections, the primary antibody was omitted. Evaluation was performed using a Zeiss LSM 710 Confocal Microscope. Quantitative analysis of the inflammatory cells was performed in digital pictures taken under the confocal microscope using x10 objective magnification.

Additional staining of T cells (similar protocol) was performed in free-floating whole mount corneas, aiming to study their distribution with respect to corneal blood vessels.

Treatment protocol

Based on the histological results, showing a massive accumulation of T cells mainly in the limbus and cornea of neovascularized eyes, we tested the beneficial effect of tacrolimus (Prograph, Teva), either as a post-exposure prophylactic treatment (at 72h), or as a symptomatic therapy given after the clinical manifestation of NV at 4weeks. The drug was given by a sub-conjunctival injection (50µl, 5mg/ml) once a week for 3weeks. A group of animals displaying a similar corneal NV served as untreated control (n=8/group). The efficacy of the treatment was evaluated by clinical observations, including pachymetry. At the end of the experiment, animals were euthanized and their eyes were processed for histology and immunohistochemistry of T cells.

Statistical analysis

Results are expressed as mean ± SEM. *P* value ≤0.05 was considered statistically significant. The quantitative data were subjected to one or two-way analysis of variance (ANOVA) or to student t-test. Whenever statistical significance was observed, multiple comparisons were carried out using the Bonferroni, simple main effect contrasts analysis for interaction (parametric data).

Results

Clinical observations

As previously described [2,3], typical symptoms of SM ocular toxicity were observed a few hours after exposure, developing into severe inflammation of the anterior segment (Figure 1A) and corneal erosions. The erosions, reflecting epithelial damage, healed spontaneously within a week, yet, the inflammatory response, although attenuated at 1W (Figure 1B), sustained for weeks after the initial exposure (Figure 1C). Consistent with our previous data, corneal neovascularization associated with LSCD, was detected as early as two weeks after exposure in 62% of the exposed eyes and in 88% at four weeks (Figure 1C). The diagnosis of LSCD in the clinically impaired eyes was confirmed by impression cytology and histology as previously described [3,27].

Pachymetry measurements in the superior and inferior peripheral regions and in central cornea (Figure 1D) demonstrated the dynamic pattern of the edema, reflecting inflammation, during the acute and late phases (up to 4weeks), with a peak at the first week and a lower magnitude chronic inflammation later on. Interestingly, the superior limbal region was thicker, compared to the inferior limbus (Figure 1D). A quantitative analysis of blood vessels' length, reflecting clinical deterioration with time, is demonstrated in E.
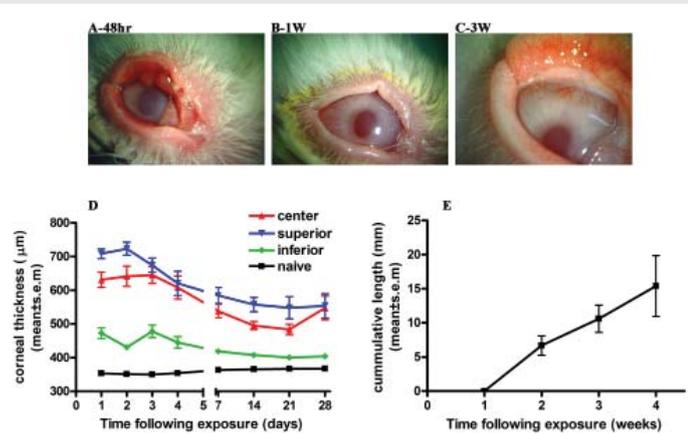
Figure 1: Clinical manifestation of ocular injuries following SM exposure in rabbits showing the inflammatory response at 48 h (A), 1 week (B) and 3 weeks (C, growth of blood vessels), peripheral and central cornea thickness reflecting edema (D) and length of blood vessels during the delayed phase in the clinically impaired eyes (E). The chronic inflammation in the exposed eyes is expressed by higher thickness values, compared to naïve. Note the lower pachymetry values found in the inferior peripheral cornea, compared to superior and central regions in D and the gradual increase in NV length representing clinical deterioration and visual impairment. The quantitative data represent the average of measurements in at least eight eyes at each time point.

### Histological characterization of the cellular inflammatory response

Histological examination of H&E stained sections of naive eyes showed a few resident inflammatory cells only in the limbal stroma, near blood vessels (Figure 2A,B). After exposure, a massive cellular infiltration was observed in the limbal stroma, starting at one week, ranging from mild to a massive reaction. The severity of the inflammatory infiltration during the late pathology was in relation with the clinical status, mainly with the extent of NV. The invading cells in the limbus were often associated with abnormal epithelium (Figure 2E). In neovascularized eyes, inflammatory cells were also detected in the cornea, accompanying growing blood vessels (Figure 2C–F).

Quantitative analysis of the number of infiltrating cells in limbal stroma supports these observations (Figure 3), showing a significant elevation already at one week ($p<0.01$, compared to naïve) which became more pronounced at 2–4weeks ($p<0.001$). Further identification of the specific type of the infiltrated cells was performed by immunohistochemistry as described below.

Characterization of infiltrating cells by immunohistochemistry

The infiltrated cells were identified by specific markers for neutrophils, T cells, APCs and macrophages.

Neutrophils were normally absent in limbal and corneal stroma (Figures 4A,D). At one week–post exposure, infiltration of neutrophils was observed in the limbus (Figure 4B) and remained, in the clinically impaired eyes for at least 2–4weeks (Figure 4C). In the central cornea, neutrophils were rarely detected at one week (Figure 4E), yet in eyes displaying NV, their number increased at 2–4weeks (Figure 4F). DAPI staining
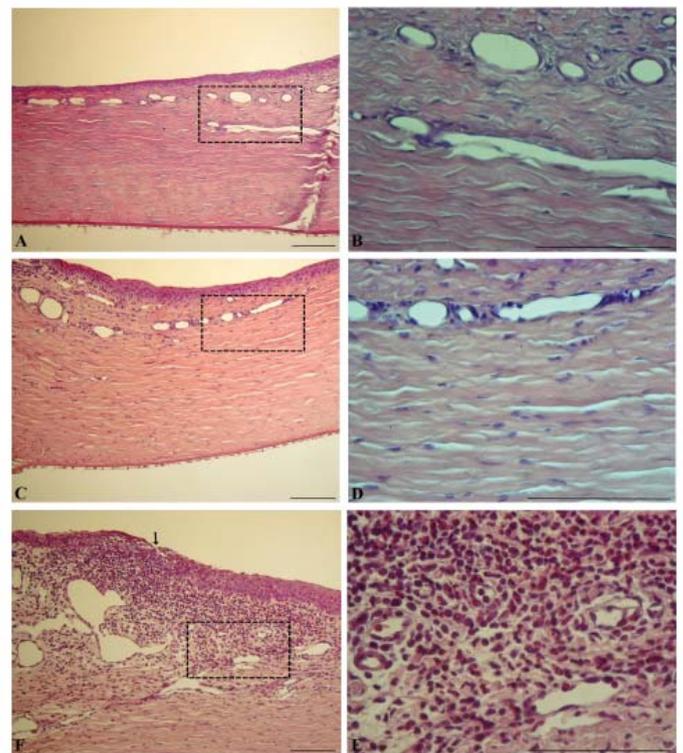
Figure 2: Histology of the limbus in naïve (A-B), one week (C-D) and 4 weeks (E-F, clinically impaired eye) following SM exposure in rabbits. The impaired epithelium is shown in E (arrow). Area of limbal stroma in insert is shown in higher magnification in B, D, and F. Note the pronounced inflammatory infiltration in the limbus in E and F. Bar scale indicates 100 μm.
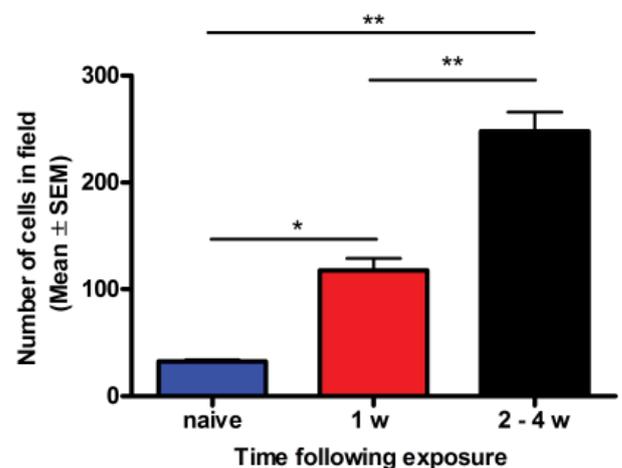
Figure 3: Quantitative analysis of the cellular infiltration in the limbus of naïve and SM exposed rabbits, indicating a significant increase at 1 week (p<0.01, compared to naïve) and at 2-4 weeks (p<0.001, compared to naïve and 1W). Analysis was performed on pictures taken from H&E stained sections. Averages are based on at least four eyes per group.

(showing more cells) revealed that neutrophils composed only part of the infiltrating cells in the limbus.

Few sporadic APCs, characterized by high expression of MHC class II, were observed in the limbus of naïve eyes (Figure 5A,B). At one week after exposure, a focal infiltration of APCs was observed in the limbus and their density was pronounced in neovascularized eyes at 2–4W (Figure 5C). APCs were seen in

the cornea (Figures 5 D-F) and their morphology in clinically impaired eyes was elongated with numerous projections (Figure 5F), supporting their identification as dendritic cells.

T cells, detected as CD43 positive cells were observed in the limbus at one week in most of the eyes and their number increased at least up to 4weeks (Figure 6A-C). The T cells, seen near the basal layer of the epithelium, were relatively large, displaying variable morphology, either elongated or rounded and their presence was pronounced in neovascularized eyes (Figure 6C). In the cornea (Figure 6D-F), migrated T cells were seen starting from one week after exposure, their number became significant at 2-4weeks and appeared in the central cornea of neovascularized eyes (Figure 6E). A close relationship was found between the number of T cells and growth of blood vessels. Moreover, their location was adjacent to blood vessels, as was seen in whole-mount flat corneas (Figure 6F).

Macrophages expressing RAM11 were not detected, either in naïve or in exposed eyes in both the limbus and cornea.
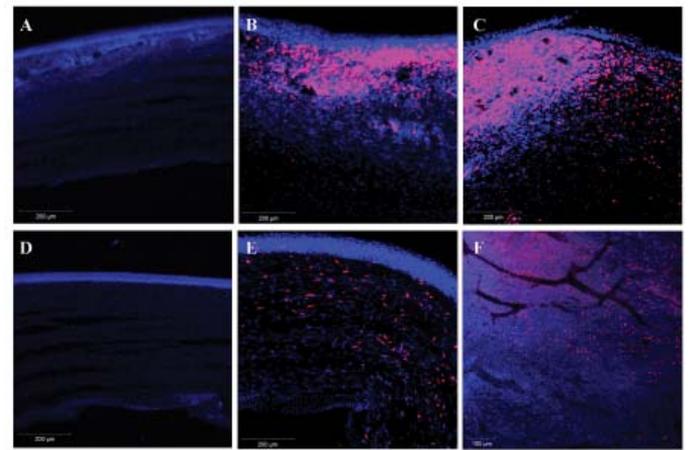
Figure 6: Immunofluorescence staining (pink) with anti-CD43 for T cells in the limbus (A-C) and cornea (D-F) in naïve (A, D) and one (B) and 2-4 weeks (C, E-F, clinically impaired eyes) following exposure of rabbits' eyes to SM vapor. Note the migration of T cells into the basal layers of limbal epithelium in B. The accumulation of T cells nearby pathological blood vessels is shown in whole mount cornea in F. Bar scale indicates 200 μm in A-E and 100 μm in F.
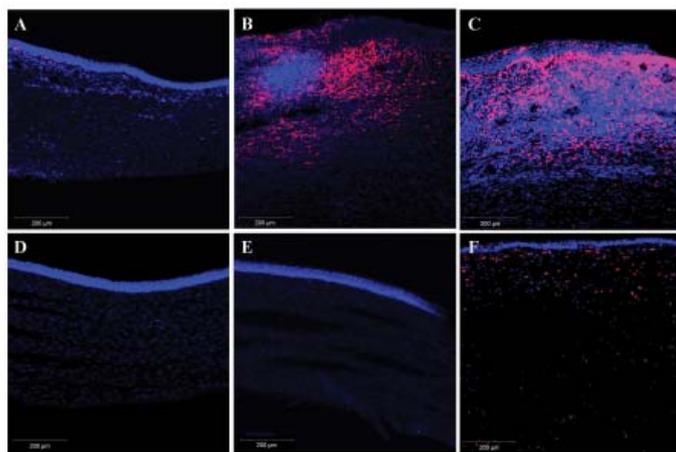
Figure 4: Immunofluorescence staining (pink) with the anti-neutrophil defensing 5 Ab (NP5 mAb) for neutrophils in limbus (A-C) and cornea (D-F) of naïve (A,D) and one (B,E) and 4 weeks (C, F, clinically impaired eye) following exposure of rabbits' eyes to SM vapor. Bar scale indicates 200 μm.
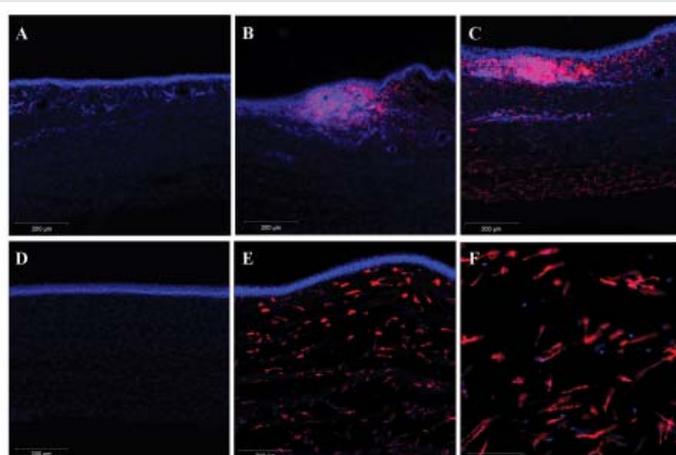
Figure 5: Immunofluorescence staining (pink) with anti-MHC class II Ab for APCs in the limbus (A-C) and cornea (D-F) of naïve (A, D) and one (B, E) and 2-4 weeks (C, F) following exposure of rabbits' eyes to SM vapor. Note the focal infiltration in the limbus of neovascularized eyes in C and the dendritic morphology of the cells in F. Bar scale indicates 200 μm in A-E and 100 μm in F.

The negative staining for macrophages in the present study was not due to technical difficulties since a positive staining was obtained in control rabbit lung sections, using the same protocol (data not shown).
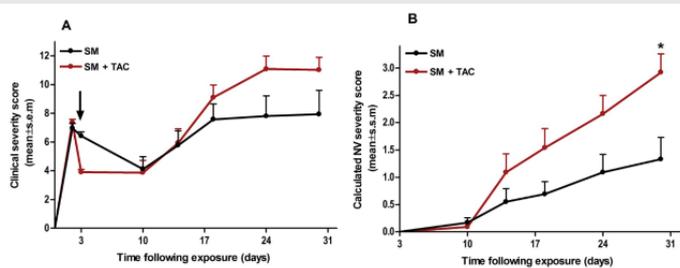
Treatment with tacrolimus

Based on our findings, showing the accumulation of T cells in the limbus and cornea beginning at one week, and their possible role in the development of corneal NV, and based on the literature, we evaluated the beneficial effect of the anti-inflammatory macrolide, tacrolimus. The drug is known as a T cells suppressor and was tested in two different regimes, as a post-exposure early treatment and at four weeks after exposure as a symptomatic therapy.

Early post-exposure treatment with tacrolimus

Post-exposure preventative treatment with tacrolimus was tested before the appearance of NV, starting at 72h, once a week for 3 weeks, compared to the exposed untreated group. The clinical observations (Figure 7) demonstrated that the treatment had no beneficial effect and some of the clinical parameters were even more severe in the treated eyes. The extent of blood vessels in the treated group was significantly larger compared to the un-treated control group ($p < 0.05$). In addition, the density of T cells was similar in the two groups (data not shown).

Symptomatic treatment with tacrolimus

Tacrolimus was administered, beginning at 4weeks post-exposure for 3weeks, to eyes displaying NV and the effect was compared to a group of untreated control that displayed a similar pathology. The clinical results, presented in Figure 8, demonstrate that tacrolimus did not have any beneficial effect on any of the clinical parameters (NV-A, corneal thickness-B), compared to the exposed untreated group. Moreover, with respect to neovascularization, the treated eyes even displayed exacerbation of the clinical symptoms.

Figure 7: The effect of an early sub-conjunctival injection of tacrolimus on the development of late injury in SM-exposed eyes. The 3 weeks treatment with tacrolimus (5 mg/ml, once a week) started at 72 h post-exposure. Note the aggravation in the clinical parameters in the treated group (P<0.05 in the neovascularization score). Average is based on at least six eyes per group at each time point.
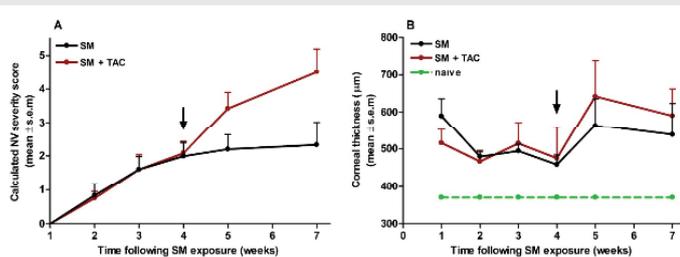
Figure 8: The effect of symptomatic sub-conjunctival injection of tacrolimus (5 mg/ml) on corneal blood vessels (A) and corneal thickness (B) in SM-exposed eyes. The 3 weeks treatment started at 4 weeks post-exposure (arrow, once a week). Note the exacerbation in the NV severity score and corneal edema following treatment with tacrolimus. Average is based on 8 eyes per group at each time point.

Immunohistochemical examination of T cells in corneas taken at the end of the experiment (7weeks) revealed that the treatment had no effect on the accumulation of T cells in the limbus and cornea (data not shown).

Discussion

The focus of the present study was the classification of the inflammatory cells in the ocular surface following SM exposure in rabbits, and based on the results, evaluation of a potential therapy. The results show that neutrophils, dendritic cells and T cells infiltrated in large quantities to the limbus and cornea following SM exposure and that T cells accumulated adjacent to pathological blood vessels in the cornea in eyes displaying the late injury. These findings directed us to evaluate the effect of treatment with the anti-inflammatory T cell suppressor tacrolimus. However, tacrolimus as a single anti-inflammatory drug was not beneficial and even worsened the late pathology symptoms, either when administered as a preventative or as a symptomatic treatment. Moreover, tacrolimus had no effect on the inflammatory clinical score, as well as on the infiltration of T cells.

Management of LSCD in general and specifically in SM injury, is a challenge from both mechanistic and treatment aspects [28]. Studying the mechanism of SM-induced late ocular injury we have previously shown that the delayed death of corneal limbal epithelial stem cells was derived indirectly from secondary processes in the limbus such as inflammation, creating a pathological microenvironment for the stem cells. This is different from other models of LSCD following a chemical ocular burn, in which limbal damage, expressed by clinical manifestations of stem cell deficiency, develops shortly after the trauma. It is accepted that the first step in the management of LSCD is to control the ocular inflammation aiming to prevent the development of pathological microenvironment for the stem cells [29]. Indeed, in our model, treatment with topical steroids was beneficial in reducing and postponing the occurrence of the SM induced late pathology in both preventative or symptomatic topical treatment [3,15,30]. However, the steroids were not sufficient to block completely the inflammation and consequently the development of the late pathology [3,10].

The inflammatory cascade

The histological observations in H&E stained sections confirmed our previous results, demonstrating a pronounced focal cellular infiltration into the limbus, starting at one week post-exposure and persisting in eyes displaying the late injury (clinically impaired). In the present study, using immunohistochemistry, we characterized some of these inflammatory cells. As expected, neutrophils, the first line of the inflammatory response, were observed in the limbus at one week accompanied by an increased number of antigen-presenting cells, morphologically defined as dendritic cells and by the migration of T cells. The dendritic cells are strong stimulators of the immune system and key players in T cell activation [31,32]. The contribution of the dendritic cells to the pathogenesis of corneal and ocular surface inflammatory diseases including corneal NV is well established [18,33]. The T cells accumulated in aggregates in the limbal stroma (the stem cell niche) and nearby pathological corneal blood vessels, as observed in whole mount flat corneas, pointing out towards the close relationship between T cells and corneal pathology.

Macrophages are the main component of the inflammatory response in general and enhance corneal NV, associated with inflammation in different experimental models, such as sutures and alkali burn [24]. Nevertheless, in the present study, macrophages were not detected in spite of the inflammatory reaction and the corneal NV, neither by morphological criteria in H&E stained sections, nor by immunohistochemistry with RAM11 Ab. Our results contradict other reports on CD68 positive macrophages in ocular mustard gas keratopathy (MGK) that represent an ongoing chronic inflammation in the chronic and delayed-onset MGK [5]. The lack of positive staining for macrophages in our study may be due to the use of a different antibody (RAM11, used to label rabbit macrophages), compared to F4/80 or CD68 used by others [5,34], or due to alternative inflammatory cascade.

Treatment with tacrolimus

The accumulation of T cells in the clinically impaired eyes and their accumulation near pathological corneal blood vessels, led us to evaluate the therapeutic potential of tacrolimus, a potent T cell suppressor. Growing evidence indicates that macrolides inhibit the inflammatory activities of the innate and adaptive immune systems, probably due to inhibition of the nuclear translocation of nuclear factor-kB (NF-kB)

and activator protein-1factor, which results in the reduced formation of pro-inflammatory cytokines [34]. Inhibition of T cells by tacrolimus may therefore lead to the reduction of the release of inflammatory mediators and decreased stimulation of additional inflammatory cells.

Yet, we could not find any decrease in the inflammatory response, i.e. clinical inflammatory parameters or cellular infiltration, following treatment with tacrolimus, in spite of its expected anti-inflammatory properties and in contrast to the beneficial effect of anti-inflammatory drugs such as dexamethasone in our model [15]. The lack of efficacy was demonstrated when tacrolimus was given during the acute phase as a preventative treatment or at 4weeks as a symptomatic treatment against existing NV. Our results, and in particular those of the symptomatic therapy, are different from other reports in the literature in other models of ocular surface diseases, showing attenuation of inflammatory parameters as well as regression of blood vessels. Topical 0.02% tacrolimus ointment was effective in treating ocular surface inflammatory diseases and 0.1% tacrolimus eye drops rapidly inhibited the activity of dendritic cells [35,36]. A long-term beneficial effect of tacrolimus suspension eye drops (0.05%) was shown on alkali burn-induced corneal NV in rats [24,25] showed an anti-angiogenic effect of tacrolimus in rabbits, following a sub-conjunctival injection or a topical treatment with eye drops, using a similar dose of tacrolimus but in a different experimental model for corneal NV. The authors showed that the anti-angiogenic effect of tacrolimus was due to inhibition of the influx of macrophages to the inflammatory site and consequently reduction in the tissue level of VEGF. The potency of tacrolimus in reducing corneal NV in Park's study was comparable to that of bevacizumab. Nevertheless, it is worth mentioning that we also showed a beneficial effect of bevacizumab in reducing NV in our model (Kadar 2014), in contrast to the lack of efficacy of tacrolimus. Thus, the absence of macrophages in our study, as well as the lack of clinical improvement of tacrolimus may point out towards a different pathological mechanism for corneal NV in these two models.

Hence, although tacrolimus can act on various components of the inflammatory cascade, in our study it failed to decrease clinical inflammatory parameters (i.e. edema) and the migration of T cells and subsequently failed to reduce the NV. Moreover, some of the clinical parameters were exacerbated in the treated eyes. In accordance, ocular side effects of tacrolimus were reported after topical application of tacrolimus in humans and animals. These included transient blurring, burning sensation and delayed epithelial healing [37]. Pro-inflammatory side effects of tacrolimus were reported also by Park, et al., (2015), despite its beneficial anti-angiogenic effect. The authors postulated that tacrolimus administration provoked more macrophage infiltration and that injecting the drug directly into the sub-conjunctiva may induce an inflammatory effect on some cytokines and therefore, it is not used routinely in the clinics in humans. Thus, it is possible that the sub-conjunctival injection in our study aggravated an inflammatory ocular reaction leading to the late pathology.

A limitation of the current study is that the efficacy of tacrolimus was tested only as a single medication while in the clinic it is usually followed or given in combination with other anti-inflammatory drugs.

In summary, the chronic inflammatory cascade in SM exposed eyes consists of an increased number of neutrophils, accumulation of dendritic cells and migration of activated T cells towards the limbus and later on to the cornea. T cells were observed in neovascularized eyes in focal aggregates in the limbus and adjacent to pathological corneal blood vessels. These findings indicate a possible role in the creation of a pathological niche for the epithelial stem cells as well as in corneal angiogenesis in our model of SM-induced delayed LSCD. Nevertheless, anti-T cell therapy with tacrolimus as a single drug did not ameliorate the clinical symptoms and was not sufficient to prevent or reduce the inflammatory response as well as the corneal neovascularization following ocular SM exposure. It is suggested that a combination of anti-inflammatory drugs with different modes of action may be more relevant in treating SM-induced ocular injury.

Declaration of interest

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

Acknowledgement

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