Evaluation of the expression of T helper lymphocytes markers associated with Th1, Th2, Th17, and Treg cells in primary pterygium biopsies

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Abstract

Introduction: Various proinflammatory cytokines and growth factors have been implicated in the pathogenesis of pterygium (Pt); however, the effect of cytokines produced by helper T lymphocytes (Th) has been poorly studied. To date, only interleukin 4 (IL-4) from Th lymphocytes has been linked to Pt recurrence. Therefore, this study aimed to evaluate the expression of Th cytokines and Th transcription factors in primary Pt. Methods: Pt biopsies were obtained from 28 eyes of 28 Mexican patients undergoing primary excision of Pt with conjunctival autograft. Conjunctival biopsies of eight patients undergoing cataract surgery were used as the control group. Gene expression of Th cytokines - interferon gamma (IFN-γ), interleukin (IL)-13, IL-17, IL-10-, as well as gene expression of Th transcription factors T-bet, GATA3, Foxp3, and RORγt, was analyzed by real-time RT-PCR. Results: We found decreased expression of markers T-bet, GATA3, Foxp3, and RORγt, was analyzed by real-time RT-PCR. Results: We found decreased expression of markers T-bet, GATA3, Foxp3, and RORγt, was analyzed by real-time RT-PCR. Results: We found decreased expression of markers T-bet, GATA3, Foxp3, and RORγt, was analyzed by real-time RT-PCR. Results: We found decreased expression of markers T-bet, GATA3, Foxp3, and RORγt, was analyzed by real-time RT-PCR. Results: We found decreased expression of markers T-bet, GATA3, Foxp3, and RORγt, was analyzed by real-time RT-PCR. Results: We found decreased expression of markers T-bet, GATA3, Foxp3, and RORγt, was analyzed by real-time RT-PCR. Results: We found decreased expression of markers T-bet, GATA3, Foxp3, and RORγt, was analyzed by real-time RT-PCR. Results: We found decreased expression of markers T-bet, GATA3, Foxp3, and RORγt, was analyzed by real-time RT-PCR. Results: We found decreased expression of markers T-bet, GATA3, Foxp3, and RORγt, was analyzed by real-time RT-PCR. Results: We found decreased expression of markers T-bet, GATA3, Foxp3, and RORγt, was analyzed by real-time RT-PCR. Results: We found decreased expression of markers T-bet, GATA3, Foxp3, and RORγt, was analyzed by real-time RT-PCR. Results: We found decreased expression of markers T-bet, GATA3, Foxp3, and RORγt, was analyzed by real-time RT-PCR. Results: We found decreased expression of markers T-bet, GATA3, Foxp3, and RORγt, was analyzed by real-time RT-PCR. Results: We found decreased expression of markers T-bet, GATA3, Foxp3, and RORγt, was analyzed by real-time RT-PCR. Results: We found decreased expression of markers T-bet, GATA3, Foxp3, and RORγt, was analyzed by real-time RT-PCR. Results: We found decreased expression of markers T-bet, GATA3, Foxp3, and RORγt, was analyzed by real-time RT-PCR. Results: We found decreased expression of markers T-bet, GATA3, Foxp3, and RORγt, was analyzed by real-time RT-PCR. Results: We found decreased expression of markers T-bet, GATA3, Foxp3, and RORγt, was analyzed by real-time RT-PCR. Results: We found decreased expression of markers T-bet, GATA3, Foxp3, and RORγt, was analyzed by real-time RT-PCR. Results: We found decreased expression of markers T-bet, GATA3, Foxp3, and RORγt, was analyzed by real-time RT-PCR. Results: We found decreased expression of markers T-bet, GATA3, Foxp3, and RORγt, was analyzed by real-time RT-PCR. Results: We found decreased expression of markers T-bet, GATA3, Foxp3, and RORγt, was analyzed by real-time RT-PCR. Results: We found decreased expression of markers T-bet, GATA3, Foxp3, and RORγt, was analyzed by real-time RT-PCR. Results: We found decreased expression of markers T-bet, GATA3, Foxp3, and RORγt, was analyzed by real-time RT-PCR. Results: We found decreased expression of markers T-bet, GATA3, Foxp3, and RORγt, was analyzed by real-time RT-PCR. Results: We found increased expression of IL-17 and Foxp3 in Pt samples suggests the presence of IL-17+ Foxp3+ Th lymphocytes, a subset of the Th population with the ability to suppress T cell proliferation and promote tumor progression. Therefore, IL17+ Foxp3+ Th cells may be involved in the occurrence and growth of Pt. Conclusions: The increased expression of IL-17 and Foxp3 in Pt samples suggests the presence of IL-17+ Foxp3+ Th lymphocytes, a subset of the Th population with the ability to suppress T cell proliferation and promote tumor progression. Therefore, IL17+ Foxp3+ Th cells may be involved in the occurrence and growth of Pt.

Key words: Primary pterygium. T helper cells. Cytokines. T cells markers.

Evaluación de la expresión de marcadores asociados a linfocitos T cooperadores del tipo Th1, Th2, Th17 y Treg en biopsias de pterigión primario

Resumen

Introducción: Diversas citocinas pro-inflamatorias y factores de crecimiento han sido relacionados con la patogenia de pterigión (Pt); sin embargo, el efecto de las citocinas producidas por los linfocitos T cooperadores (Th) ha sido pobremente estudiado. Hasta la fecha, sólo la interleucina 4 (IL-4) de los linfocitos Th se ha relacionado con la recurrencia del Pt. Por lo tanto, este estudio se llevó a cabo para evaluar la expresión de las citocinas Th y los factores de transcripción Th en Pt.
Introduction

Pterygium (Pt) is considered a benign epithelial tumor of the cornea whose main feature is focal alteration of the sclerocorneal limbus. Its presence is associated with the symptoms of eye discomfort such as burning, irritation, tearing, and foreign body sensation. Vision is usually affected in advanced stages as a result of induced astigmatism and obstruction of the visual axis. On rare occasions and only in severe cases symblepharon appears, limiting eye mobility and producing diplopia. It is more prevalent in the equatorial zones and its occurrence is directly related to exposure to ultraviolet radiation, inflammation, and other irritating factors. Although its etiology has not been clarified, it is known that participation of the p53 gene, apoptosis disturbance, action of collagenases, and angiogenesis, promote its development.

According to the American Academy of Ophthalmology, Pt is classified according to its occurrence into (a) PRIMARY Pt: without previous surgery and (b) recurrent Pt: history of one or more previous surgical treatments (regardless of the method used). According to its extension, Pt is divided into (a) Grade I: involves the sclerocorneal limbus, invading up to 1 mm, (b) Grade II: invades cornea more than 1 mm, without reaching the pupillary border, (c) Grade III: reaches the pupillary border, and (d) Grade IV: extends beyond the pupillary margin.

Histologically, Pt is defined as a benign fibrovascular hyperplasia of the bulbar conjunctiva that invades the cornea, with leukocyte infiltration that includes helper T lymphocytes (Th) or CD4+ Th. Th lymphocytes are capable of differentiating into distinct subpopulations, characterized by the cytokines profile they produce and the transcription factors that drive their differentiation, which together are called Th markers. Specific markers for Th1 lymphocytes are interferon gamma (IFN-γ) and transcription factor T-bet; for Th2, interleukin 13 (IL-13) and transcriptional factor GATA-3; for Th17 subpopulation, IL-17 and RORγt factor; and for Tregs, IL-10 and Foxp3.

The cytokine profile secreted by each Th lymphocyte subpopulation is associated with different biological activities, and even with specific pathologies. For example, Th1 cells are responsible for cell-mediated immune responses, whereas Th2 cells are responsible for humoral immunity. When an excessive Th1 response occurs in any tissue, it results in intense tissue damage, whereas an excessive Th2 response is associated with atopy and hypersensitivity. Regarding Th17 subpopulations, its role in promotion and enhancement of inflammatory response has been described, and they have been involved with autoimmunity phenomena.

Finally, several studies have identified Treg cells as immune regulators in a large number of inflammatory and autoimmune diseases including asthma, multiple sclerosis, and type I diabetes.

Despite the current knowledge about Th cells subpopulations and their biological effects in different pathologies, the effect of Th cytokines in the pathogenesis of Pt has been poorly analyzed. Recent reports suggest that IL-4 expression could be associated with Pt relapse.

Although benign in nature, Pt is considered a public health concern due to its high prevalence and the costs derived from its attention, so studies that contribute to the understanding of its pathogenesis are desirable because they allow the identification of therapeutic targets and the design of effective preventive and therapeutic strategies. Since the expression of Th cytokines and their relationship with Pt has not been elucidated, in the present work, we review the expression of Th lymphocyte markers in primary Pt biopsies to add knowledge about the pathogenesis of this clinic entity.
Methods

Study location and ethical considerations

The project was performed at Hospital de Especialidades del Centro Médico Nacional de Occidente del Instituto Mexicano del Seguro Social and was authorized by the Local Committee of Health Research 1301 of said hospital with registry number R-2012-1301-43 since it fulfilled the methodological ethics and research requirements. It is important to emphasize that this project was compliant with the ethical guidelines laid down in the Declaration of Helsinki.

Biopsy collection

We included in the study healthy conjunctiva biopsies (control group, n = 8) obtained from subjects undergoing elective cataract surgery, and primary Pt biopsies (n = 28, seven biopsies for each degree of primary Pt extension - Grades I to IV) of subjects undergoing monocular excision of Pt with conjunctival autograft placement (Fig. 1). Before surgery, the informed consent of all the patients was obtained after extensive explanation of the study objectives and its scope. The biopsies obtained during surgery were immersed in RNA later solution (Applied Biosystems, CA, USA) to ensure mRNA integrity. After incubation for 12 h at 4°C, biopsies were stored at −80°C until use.

Total RNA extraction

Total RNA extraction from Pts or conjunctival biopsies was performed using the Trizol reagent (Invitrogen, CA, USA). Said reagent is defined as a monophasic solution of phenol and guanidine thiocyanate and constitutes an improvement of the one-step total RNA extraction method proposed by Chomczynski and Sacchi11. Total RNA extraction started with the addition of 250 μl of Trizol to 200 mg of tissue. After a brief incubation on ice (1 min), the sample was homogenized with an automatic homogenizer. Subsequently, 150 μl of chloroform was added to the homogenate, and after incubation for 3 min at room temperature, the sample was centrifuged at 18,000 rcf/20 min/4°C to separate the sample in three phases: phenolic, intermediate, and aqueous. The aqueous phase was collected. RNA was obtained from the aqueous phase by precipitation, adding isopropanol (1:4) and then incubating for 24 h at −20°C. After incubation, the sample was centrifuged at 18,000 rcf/15 min/4°C to obtain the RNA pellet located at the bottom of the microtube. RNA pellet was rinsed 3 times with 75% ethanol. Ethanol was removed by decantation and RNA was solubilized in RNase-free water. The RNA obtained was quantified by spectrophotometry at a 260 nm wavelength and its quality was verified by means of the 260/280 ratio.

Determination of Th markers gene expression by real-time RT-PCR

For the analysis of the expression of genes and Th markers, a single-step method of retro transcription-polymerase chain reaction (RT-PCR) was used (Invitrogen, CA, USA). A volume of 2 μl of the total RNA samples from the study groups was taken at a concentration of 8 ng/μl, which was subjected to real-time RT-PCR reaction using specific probes for Th cytokine genes (IFN-γ, IL-10, IL-13, and IL-17), as well as for Th
transcription factors genes (T-bet, GATA-3, Foxp3, and RORγt) (Applied Biosystems, NJ, USA). The reaction included: taqMix, DyeRox, and Reaction Mix 2x. Gene expression was normalized with 18s ribosomal RNA expression. The temperature conditions in the PCR reaction were the following: 30 min/48°C, 10 min/95°C, and 40 cycles of 15 s/95°C alternated with 1 min/60°C. The PCR reaction and the detection of the amplification products were carried out in the OneStep Plus thermal cycler (Applied Biosystems, NJ, USA). Relative quantification of gene expression was performed by the method of \(2^{-\Delta\Delta CT}\) using the control group for internal calibration

Statistical analysis

Data with non-normal distribution were analyzed using the non-parametric tests Kruskal–Wallis, Dunn’s, and Mann–Whitney U-test. Data with normal distribution were analyzed by one-way ANOVA. Post-hoc tests, Holm-Sidak, and Dunnett were used to determine statistical significance. The results are presented as mean ± standard error. Statistical significance was defined as \(p < 0.05\).

Results

To determine the influence of Th lymphocytes subsets on primary Pt, gene expression of several specific markers of Th1 (IFN-γ and T-bet), Th2 (IL-13 and GATA-3), Th17 (IL-17 y RORγt), and Treg (IL-10 y Foxp3) was determined in Pt biopsies obtained from patients with different degrees of Pt extension.

As depicted in figure 2, the expression of Th cytokines showed a different pattern for each subpopulation, especially for Th2 and Th17 subpopulations. The Th2 cytokine IL-13 showed a significant decrease compared to the control group, in an extension-dependent manner (the greater the extension, the lower the expression). Regarding the expression of IL-17 in the Th17 subpopulation, the expression increased compared to the control, especially in Pt Grades I and IV. IFN-γ and IL-10 expression (Th1 and Treg cytokines, respectively) did not show significant differences between study groups.

Regarding the expression of Th transcription factors (Fig. 3), an increase in the expression of T-bet and Foxp3 factors was observed, which decreased as Pt extension increased. GATA3 expression significantly decreased in a pattern that mimics IL-13 expression. Finally, RORγt expression did not show a significant difference between groups.

It is important to highlight that unlike Th2 markers where there is concordance between the levels of their cytokine (IL-13) and their transcription factor (GATA3), the expression pattern of the remaining Th markers was not coincident. Interestingly, an over-expression of IL-17 (Th17 marker) and Foxp3 (Treg cell marker) was observed in Pt biopsies compared to healthy conjunctiva biopsies (\(p < 0.0001\)).

Discussion

Since current knowledge about the expression of Th cytokines in Pt is limited, we decided to evaluate the expression of Th markers in primary Pt biopsies. As the
results show, the expression of T-bet, GATA3, RORγt, IFN-γ, IL-13, and IL-10 genes was found to be decreased in Pt, while the expression of IL-17 and Foxp3 increased up to 6 times in Pt biopsies compared to healthy conjunctiva (p < 0.0001); this increase was dependent on Pt extension (greater extension meaningless expression).

In Grade I Pt, it can be observed that the expression of IL-13 is at the same level as in healthy conjunctiva. Some studies suggest that IL-13 is the predominant Th2 cytokine in the normal ocular surface, which is why we find a basal expression in the tissues that changes when Pt extension increases. On the other hand, it has been described that Pt biopsies obtained from atopic and non-atopic patients show a significant increase in IFN-γ, which agrees with the results observed in our samples. It is important to emphasize that although IFN-γ expression is strongly diminished in all degrees of extension of Pt, we can observe that in biopsies corresponding to Grades I, III, and IV, the expression of the transcriptional factor Th1 (T-bet) is increased. This suggests that in the Pt microenvironment there is the main stimulus of Th1 (IL-12) differentiation but not enough to induce polarization. In contrast, the expression of Th2 transcriptional factor (GATA3) is diminished in the same Pt biopsies, which may be due to the inhibitory effect of T-bet on the expression of GATA3. The increased expression of IL-17 and Foxp3 in Pt samples suggests the presence of IL-17+ Foxp3+ Th lymphocytes a subset of the recently described Th population with the ability to suppress T cell proliferation and promote tumor progression. In previous studies, in patients with primary and relapsing Pt, it was found that both showed an elevated level of IL-17 in the tear film, so it was suggested that this could be associated with its occurrence and recurrence.

IL-17+ Foxp3+ Th cells have been identified in various human diseases such as inflammatory bowel disease, colonic tumors, periodontitis, psoriasis, and rheumatoid arthritis. Characteristically, these cells conserve the immunosuppressive activity of Treg lymphocytes, while they acquire effector capacity due to the production of IL-17. It is known that the microenvironment in which the precursor Th lymphocytes (naïve) is found determines the phenotype of the differentiated Th lymphocyte; however, it is also important to consider that effector Th lymphocytes can modify their phenotype in response to surrounding stimuli. IL-17+ Foxp3+ Th lymphocytes emerge on stimulation of IL-17 and IL-23 on Treg lymphocytes. We can observe that in the degrees of extension II and III the expression of IL-17 is diminished, which could be explained by the phenomenon described above, that is, in these degrees of Pt extension the microenvironment is not ideal for differentiation of IL-17+ Foxp3+ cells.

In our study, we observed the inhibition of Th1 and Th2 cytokines (particularly IL-13), which is consistent with the supposed suppressive activity of IL-17+ Foxp3+ Th lymphocytes, and at the same time, we documented that the expression of these markers was associated with the progression of Pt, which coincides with the tumorigenic activity of this cell population. However, it is necessary to emphasize that although in this project, we identified the overexpression of IL-17 and Foxp3 in Pt biopsies, it is necessary to perform complementary studies to corroborate the presence of this cellular subpopulation, for example, by identifying the specific surface markers in cells obtained from the tissues. In conclusion, IL-17+ Foxp3+ Th lymphocytes could be involved in the development and progression of Pt; however, complementary studies are needed to confirm and extend our findings.

Ethical responsibilities

Protection of human and animal subjects. The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics committee and with those of the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Confidentiality of data. The authors declare that they have followed the protocols of their work center on the publication of patient data.

Right to privacy and informed consent. The authors have obtained the written informed consent of the patients or subjects mentioned in the article. The corresponding author is in possession of this document.

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