Chapter 6

Ophthalmological Manifestations and Tear Investigations in Systemic Sclerosis

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Additional information is available at the end of the chapter

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Abstract

Systemic sclerosis (SSc) is a chronic autoimmune disorder characterized by widespread small vessel vasculopathy, immune dysregulation with production of autoantibodies, and progressive fibrosis. There are only few reports available concerning ophthalmological complications in the course of SSc, although ocular manifestations, e.g., dry eye syndrome (DES), occurs frequently and decreases the quality of life of these patients. Vascular endothelial growth factor (VEGF), the major pro-angiogenic factor, plays a key role in the pathomechanism of SSc. Although elevated levels of VEGF in sera have already been demonstrated, VEGF analysis in tears of patients with SSc has not been performed in previous studies. VEGF in the tears of patients with SSc was found to be decreased by 20%, compared to healthy controls. The reason why the VEGF levels are not elevated in the tears of patients with SSc needs further investigations, as does the sera of the same patients. The cytokine array results revealed a shift in the cytokine profile characterized by the predominance of inflammatory mediators. Our current data depict a group of cytokines and chemokines, which play a significant role in ocular pathology of SSc; furthermore, they might function as excellent candidates for future therapeutic targets in SSc with ocular manifestations.

Keywords: systemic sclerosis, dry eye syndrome, tear, vascular endothelial growth factor, cytokine, tear sampling, total protein, enzyme-linked immunosorbent assay, cytokine array, multiplex bead assay

1. Introduction

There are few reports, mainly case reports, available concerning ophthalmological complications in the course of systemic sclerosis (SSc). Overall studies are even fewer involving

only a small number of patients, since SSc is a rare disease [1, 2]. Changes in the organ of vision are thought to be the consequences of systemic complications of scleroderma or adverse effects of the immunosuppressive treatment applied. Ocular symptoms may occur at any stage of the disease and may involve numerous ocular tissues. Their course can be clinically latent or very intensive. The most prevalent clinical manifestations of soft tissue fibrosis and inflammation in patients with SSc include increased tonus and telangiectasia of the eyelid skin. The most common lesions reported include periorbital edema, palpebral ectropion, and madarosis [3].

The most frequent ocular manifestation of SSc in our studies was dry eye syndrome (DES).

DES is a major healthcare problem because it affects the patient's quality of life. DES in SSc is believed to be caused by fibrosis-related impairment of lacrimal gland secretion, namely, the reduction of the water portion of the tear film. Furthermore, lipid layer disorder is caused by chronic blepharitis and meibomian gland dysfunction (MGD), while increased evaporation of tears from the ocular surface is the consequence of restricted eyelid mobility and the consecutive reduced blinking [2]. DES was recently redefined as a multifactorial disease of the tears and ocular surface that results in symptoms of discomfort, visual disturbance, tear film instability, and last but not least damage to the ocular surface [4]. Increased osmolality of the tear film [5] and inflammation of the ocular surface [6] are the two major characteristic features of this ocular surface disease. The most important laboratory findings [6] are increased levels of several inflammatory cytokines. Accordingly, tear cytokine levels are regarded as potential markers of inflammation in DES.

The ophthalmological manifestations in patients with SSc are frequently underestimated and not or not correctly treated. In order to better understand the ocular features and use this body fluid as a potential tool for monitoring these important biomarkers, we have turned our attention to tear investigations.

Precorneal tears as a biological fluid are very easily accessible with non- or very low-invasive methods at a relatively low cost. Tears not only lubricate the ocular surface carrying secreted molecules from corneal epithelial cells and tissues producing tear components but also represent the whole physiological status of the body. Due to the very limited amount of samples and the relative instability of the components, sample collection is a critical step in tear research and diagnostics.

Although tear analysis is of increasing interest in ophthalmology, no studies have investigated tears of patients with SSc as yet, possibly because of the technical challenge posed by the extremely small sample volumes available [7].

Quantitative determination of tear proteins is of increasing interest in ophthalmology, but a technical problem still remains due to small tear sample volumes available on the one hand and the complexity of their composition on the other [7, 8]. Tear sampling performed either directly or indirectly is definitely a major challenge or has a most significant influence on the precision and reproducibility of the analytical results as seen in the summary below.

1.1. Direct sampling methods

Direct sampling methods use microcapillary tubes [9] or micropipettes for sampling. This requires previous stimulation or instillation of different volumes of saline (100–200 μl) into the cul-de-sac and collecting after appropriate mixing. The procedure causes dilution and may not permit collection of samples from specific sites of the ocular surface [10].

Kalsow et al. investigated the tear cytokine response to multipurpose solutions in contact lens [11] wearing. Before tear collection, contact lens was removed, and then NST tears were collected from both eyes from the inferior lateral conjunctival cul-de-sac using a 10-μl flame-polished glass micropipette. The collection, a 5.5-μl tear volume, was immediately transported to a sterile 0.2-ml tube containing 49.5 μl of storage solution to produce a 1:10 tear dilution for immediate storage at −80°C [11].

Guyette et al. compared low-abundance biomarker levels in capillary-collected NST tears and washout (WO) tears of aqueous-deficient and normal patients. 10-microliter polished micropipettes were used to collect tears from the inferior marginal strip, taking special care to minimize ocular surface contact. Tear collection rate was continuously monitored. Individual NST tear samples were collected in 10-min aliquots and immediately transferred to a sterile polymerase chain reaction (PCR) tube. An equal volume of assay buffer was added, and the sample was stored at −86°C. A total of at least 6.5-μl NST tears were collected from each study participant, and each 10-min aliquot was transferred into a separate PCR tube and put in the freezer without delay. Prior to WO tear sample collection, 10-μl sterile physiologic saline solution was added to the lower conjunctiva by a digital pipette. The patient was instructed to gently close the eyes and avoid any eye movements for one minute. Tears were then collected using the same method as for NST samples, but a shorter collection time of 5 min per aliquot was used to make up the 6.5-μl minimum volume required. Tear collection volume and time were continuously monitored to measure tear collection rate [12].

There have been several research projects focusing on dry eye syndrome, and nowadays the emphasis has shifted toward the role of inflammation in the anterior surface of the eye [13]. Since inflammatory mediators originating from various ocular surface sources and the main lacrimal gland do not constitute a totally homogenous mix, the way the tears are collected will influence the resulting biomarker profile. NST tear samples from the inferior marginal strip cover a broader spectrum of the sources, whereas ST samples contain a higher proportion of the lacrimal gland secretion [14]. Explicit protein profile differences between NST and ST tears demonstrate that these two sample types are not equivalent [15, 16]. Although NST tears represent specifically the inflammatory status of the ocular surface, the volume of NST tears is limited, especially in aqueous-deficient dry eye. Even though tear sampling frequently makes use of capillaries as they are less irritating and the resulting sample is an exact representative concentration of molecules, the main limitation of the method is the volume of sample (2–3 μl) to be gained [17].

One way to increase the available tear sample volume is to add fluid (e.g., sterile saline) to the eye prior to sample collection, effectively “washing out” ocular surface molecules [18, 19].

In an experimental dry eye study, Luo et al. collected tears from mice with tear fluid washing [20]. Tear fluid washings were collected by a method previously reported by Song et al. [21]. Briefly, 1.5 μl of phosphate buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) was instilled into the conjunctival sac. The tear fluid and buffer were collected with a 10-μl volume glass capillary tube by capillary action from the tear meniscus in the lateral canthus. The 2-μl sample of tear washings was pooled from both eyes of each mouse and was stored at −80°C until zymography and enzyme-linked immunosorbent assay (ELISA) were performed.

Validity of the WO method depends on the extent to which it changes the NST tear biomarker profile. By determining tear sIgA, inducement of reflex tearing is easily detected because tear sIgA levels decrease with reflex tear flow rate [16]. Markoulli et al. found equal tear sIgA-total tear ratios in WO and NST tears, which suggests that WO tear samples do not significantly induce reflex tearing [19]. Guyette's study evaluated WO tear collection as a replacement for microcapillary NST tear collection and applied this to compare biomarker levels between aqueous-deficient (AD) dry eye and non-AD patients [12, 15, 22].

1.2. Indirect methods

An indirect method means that collection of precorneal tear film (PTF) is carried out using absorbing supports such as Schirmer test strips (STS), filter paper disks, cellulose sponges, and polyester rods. STS collection is the most commonly used method among them [23].

Acera et al. analyzed the inflammatory markers in the PTF of patients with ocular surface disease. 10 μl of tear samples was collected by a Weck-Cel sponge [24]. The concentrations of IL-1β, IL-6, and pro-MMP-9 were measured by ELISA, and the MMP-9 activity was evaluated by gelatin zymography.

Inic-Kanada et al. compared ophthalmic sponges and extraction buffers for quantifying cytokine profiles in tears using Luminex technology. They found that Luminex detection of cytokine/chemokine profiles of tears collected with Merocel sponges may be useful in clinical studies, for instance, to assess cytokine profile evaluation in ocular surface diseases [25].

Samples obtained from the Schirmer test procedure have been found to have a higher mucus, lipid, and cellular content than microcapillary (MC) samples [26]. STS also suffers from incomplete, nonuniform elution of proteins from the filter matrix [23]. Although micropipette and STS collection provide different biomarker profiles for a given donor, the correctly applied micropipette method has proved to be more consistent [27]. STS is widely applied as the volume of sample collected with this method is larger than other methods, but it can cause reflexive tearing due to irritation, which increases the volume of the samples, thus aggravating the detection of the investigated tear component(s), e.g., drug levels [9].

In comparative studies, the tears of the same patient are collected using several collection methods to determine the same biomarkers from the different tear samples.

Green-Church et al. collected tears using small volume (1–5 μl) Drummond glass MC tubes with 1.6× slit-lamp magnification. Non-reflex tears were collected from the inferior tear prism without contact with the lower lid until a total of 5 μl had been collected. During a separate visit, tear collection was performed by placing an STS over the lower lid. The lid was canthus. The subject was instructed to close his/her eyes for the 5-min test duration; the wet length was not recorded but was observed to be within normal ranges in all cases. The STS was then placed in 1.6-ml amber Eppendorf tube and stored at 4°C until analysis [27].

Lee et al. used two collection techniques for the comparative analysis of polymerase chain reaction assay for herpes simplex virus 1 detection [28]. Tear samples were collected from the lower fornix using STS for 5 min, a method adopted in a previous study of Satpathy et al. [29]. The other collection method they used was micropipetting tears, after irrigating 100-μl saline in the lower fornix, a method that was described in a previous study of Markoulli et al. [19], who validated the "flush" tear collection technique as a viable alternative to basal and reflex tear collection.

2. "Main body of the paper"

The aims of our studies were the following:

1. To select an appropriate sampling method to investigate vascular endothelial growth factor (VEGF) and cytokines in tears of SSc patients.
2. To detect VEGF in tears of SSc patients.
3. To compare VEGF levels in tears of patients with SSc to those in healthy controls.
4. To determine a wider panel of cytokines and chemokines that have a role in immunopathogenesis and inflammatory processes in tears of patients with SSc.
5. To compare the levels of identified mediators in tears of these patients and controls and to select the most significantly differing ones for further investigations.
6. To determine the selected mediators with the help of a more sensitive and specific laboratory method in tears of both patients and controls.

2.1. Patients and healthy controls

In the first study, 43 patients with SSc (40 female and 3 men) and 27 healthy controls were included. In the second study, we enrolled 9 patients and 12 controls. Mean (SD) age of the patients was 61.85 (48–74) years. SSc was diagnosed based on the corresponding international criteria. Patients were enrolled from the outpatient clinic at the Department of Rheumatology. They went through ophthalmological examination and basal tear sample collection at the Department of Ophthalmology. None of the patients had secondary Sjögren's syndrome. The healthy control groups were composed of age- and gender-matched volunteers with no

history of any autoimmune or ocular disorder. Patients did not take immunosuppressive medications at the time of the tear sampling.

Written informed consent was obtained from all patients and controls. Study protocol was approved by the local bioethics committee and followed the tenets of the declaration of Helsinki.

2.2. Tear sample collection

Unstimulated, open-eye tear samples were gently collected from the inferior temporal meniscus of both eyes, using glass capillary tubes (Haematokritkapillare, 75 μL, L 75 mm, Hirschmann Laborgerate, Germany), minimizing irritation of the ocular surface or lid margin as much as possible.

In the course of the first study, samples were collected between 11 a.m. and 16 p.m. by the same physician. Tear-secretion velocity was counted by dividing the volume of collected sample with time of secretion. Volume was calculated from the lengths of the fluid column in the capillary tube, measured with a vernier caliper, and from the known diameter of the tube. Time of tear collection was measured with a stopwatch.

In the course of the second study, tear collection was performed between 9 and 11 a.m.

Tears were transferred into low-binding-capacity Eppendorf tubes by the help of a sterile syringe and a needle, carried on dry ice to the laboratory and stored at −80°C until assessment. The samples were obtained from both eyes of each individual and were pooled due to the small volume available.

2.3. Quantification of total protein and VEGF levels in tear samples of patients with SSc

First, as a point of reference for VEGF, total tear protein concentrations were determined using the microplate method of the bicinchoninic acid (BCA) Protein Assay Kit (Pierce Biotechnology, Rockford, USA) adapted to a 384-well microplate due to the small sample amounts. The kit is a two-component, high-precision, detergent-compatible assay. Total protein concentration determination was based on color intensity measurement proportional to the peptide bound and the protein provided with the reagent set. The reaction absorbs visible light, namely, the wavelength 562 nm.

We used a human VEGF immunoassay kit by Quantikine (R&D Systems, Minneapolis, MN, USA) for the quantitative determination of VEGF in tear fluid. This assay employs the quantitative sandwich enzyme immunoassay technique.

2.4. Membrane array and multiplex bead analysis of tear cytokines in SSc

To remove cells, cellular debris, and contaminant particles, tear samples were centrifuged (10 min, 15,000 rpm, 4°C) prior to use.

Tear samples of controls and patients were used for cytokine profiling. The relative levels of 102 different cytokines were determined by Proteome Profiler Human XL Cytokine Array Kit (R&D Systems) using 50-μl samples according to the manufacturer's instructions. The pixel density in each spot of the array was determined by ImageJ software.

Alternatively, the absolute levels of MCP-1, complement factor D (CFD), IP-10, and C-reactive protein (CRP) were determined from diluted tear samples (CFD, MCP-1, and CRP, 1:10; IP-10, 1:40) by Human Luminex Performance Assays (R&D Systems) according to the manufacturer's instructions. The measurement was run on Bio-Plex 200 Systems (Bio-Rad) workstation.

2.5. Results

2.5.1. Vascular endothelial growth factor in tear samples of patients with systemic sclerosis

The average tear secretion velocity in patients was 4.53 μl/min with a median of 3.8 μl/min (1.5–25.6).

Duration of tear sample collection from patients varied between 20 and 313 s, until 5 μl, the minimally required volume was reached.

The average collected tear fluid volume was 10.4 μl (1.6–31.2) in patients and 15.63 μl (3.68–34.5) in controls.

In tear samples of patients with SSc, the average total protein level was 6.9 μg/μl (1.8–12.3), and the average concentration of VEGF was 4.9 pg/μl (3.5–8.1) in the case of basal tear secretion.

Control tears contained on average 4.132 μg/μl (0.1–14.1) protein and 6.15 pg/μl (3.84–12.3) VEGF.

2.5.2. Membrane array and multiplex bead analysis of tear cytokines in systemic sclerosis

2.5.2.1. Cytokine array results

Nonstimulated tear cytokine profiles of the control groups and patients with SSc were analyzed by cytokine array detecting 102 different cytokines. Array results revealed a shift in cytokine profile characterized by the predominance of inflammatory mediators. The following 9 out of the 102 analyzed molecules were significantly increased in tears of patients with SSc: complement factor D (CFD), chitinase-3-like protein 1 (CHI3L1), C-reactive protein (CRP), epidermal growth factor (EGF), interferon-γ-inducible protein 10 (IP-10, also called CXCL-10), monocyte chemoattractant protein-1 (MCP-1), monokine induced by gamma interferon (MIG), matrix metallopeptidase 9 (MMP-9), and vitamin D binding protein (VDBP) (**Table 1**).

Integrated density values were normalized to positive control spots and total protein content of the samples. Cytokine array data are representative of four control and four SSc samples.

Name of the cytokines and chemokines	Normalized density—patients with SSc	Normalized density—healthy controls	Significance of the difference (p)
CFD	50.35 (23.17–53.76)	22.33 (18.39–24.75)	0.002072
CHI3L1	94.41 (31.9–95.98)	31.06 (20.37–45.85)	0.000000
CRP	25.98 (15.28–53.16)	4.55 (4.35–4.66)	0.018250
EGF	53.42 (34.86–70.23)	34.04 (20.42–47.61)	0.032818
IP-10	123.42 (93.81–152.35)	21.99 (12.12–29.01)	0.000000
MCP-1	19.93 (5.38–42.44)	1.72 (1.44–2.27)	0.044726
MIG	22.85 (5.6–64.14)	3.58 (3.29–3.88)	0.033787
MMP-9	49.10 (4.24–129.04)	12.74 (10.29–17.56)	0.000068
VDBP	31.35 (11.87–64.68)	10.18 (8.3–13.84)	0.019733

Mean total protein values did not differ significantly in tears of patients and controls. Mean total protein value was 40.9239 μg/ml in tears of patients with SSc and 42.536 μg/ml in tears of healthy controls (p = 0.863604).

Table 1. Normalized densities of cytokines and chemokines in patients with SSc and healthy controls.

2.5.2.2. Multiplex cytokine bead assay results

By using the more sensitive and more specific Luminex bead assay, 4 selected molecules were determined in tears of 9 healthy controls and 12 patients with SSc.

Based on the Luminex bead results, mean CRP levels were 103.44 (3.57–359.02) μg/mg protein in tears of patients with SSc and 7.41 (0.87–18.03) μg/mg protein in tears of healthy controls.

Mean IP-10 levels were 564.78 (252.62–1107.2) μg/mg protein in tears of patients with SSc and 196.118 (101.66–514.37) μg/mg protein in tears of healthy controls.

Mean MCP-1 levels were 2626.83 (457.84–5619.4) μg/mg protein in tears of patients with SSc and 661.27 (397.87–1171.4) μg/mg protein in tears of healthy controls.

Mean CFD levels were 15.27 (5.00–35.28) μg/mg protein in tears of patients with SSc and 23.31 (5.18–106.63) μg/mg protein in tears of healthy controls.

Except for CFD all results were significant at p = 0.01 for CRP, p = 0.001 for IP-10, and p = 0.01 for MCP-1, respectively.

Values represent the mean (±SD) of the 9 control and 12 patient samples, which are the fold change of normalized cytokine levels.

The difference between total protein values of control and SSc tear samples was not significant (p = 0.37263). Mean total protein was 818.46 (779.94–1162.4) μg/ml in tears of patients and 872.46 (771.78–1359.5) μg/ml in tears of controls.

Based on both the cytokine array and the multiplex bead assay results, concentrations of IP-10 showed the most significant difference in tears of patients and controls.

2.6. Discussion

Although ocular manifestations in systemic autoimmune diseases have significant debilitating effects, tear analysis has been missing from the repertoire of investigations. Since tears represent the local homeostasis of the ocular surface better than serum, this makes tears ideal for assessing ocular pathology in the disease. There are two possible ways for cytokines to appear in the precorneal tear film. Some are locally produced and diffuse into the tear film from the corneal and conjunctival epithelia; others leak into the tear film from the conjunctival blood vessels [30]. Tear investigation is a challenging research field; though sample collection is noninvasive, it has an almost insurmountable limitation, the quantity of the sample obtainable [31].

Tear investigation studies have been performed in different ocular and systemic disorders [30, 32, 33]. Leonardi et al. assessed multiple mediators, such as cytokines, matrix metalloproteases, and angiogenic and growth factors in tears of patients with vernal keratoconjunctivitis. These analyses identified previously unreported factors in tears of patients, including MMP-3 and MMP-10 and multiple proteases, growth factors and cytokines, which may all be instrumental in the pathogenesis of conjunctival inflammation. Different molecules were identified in human tear samples that were involved in the development and maintenance of corneal neovascularization. Concentrations of the pro-angiogenic cytokines such as IL-6, IL-8, VEGF, MCP-1, and Fas ligand were determined in blood and tear samples using flow cytometry-based multiplex assay. These investigations resulted in significantly higher concentrations of pro-angiogenic cytokines in human tears compared to their concentrations in serum; furthermore highest levels were revealed in basal tear samples [30]. These findings lend further support to the importance of our current studies.

After reviewing the literature on direct and indirect tear sampling methods in various ocular and systemic disorders, we have chosen the microcapillary method for tear sampling in patients with SSc, since it is safely applicable for the collection of nonstimulated tears. In order to transfer the tear fluid from the microcapillary tube to the collection tube, we applied a sterile syringe and a needle. This tear sampling method proved to be suitable for our experiments on tear cytokines.

2.6.1. VEGF in tear samples of patients with SSc

VEGF is one of the components of normal tear fluid [34]. Vesaluoma et al. determined VEGF concentrations in healthy tears. The median VEGF concentration was 5 pg/μl (4–11) consistent with our results, as control tears contained an average of 6.15 pg/μl (3.84–12.3) VEGF [35].

They calculated the average tear fluid secretion in healthy controls, which was 8.1 μl/min (0.7–20.8), using the same tear collecting method as we did in our study. Results show that patients with SSc have significantly decreased tear secretion that could be explained by DES, which is a probable sequel of the disease or to the side effects of the therapeutic drugs [36].

Tear-secretion velocity was lower by 67% in patients with SSc than in healthy controls. The difference was significant ($p < 0.01$). The reason for this sign could be explained by the pathophysiology of the disease, namely, fibrotic processes of the lacrimal gland.

Total protein values in patients with SSc were higher by 42% than in healthy controls. This may indicate that total protein production—or simply protein concentration, since patients with SSc have a decreased tear secretion velocity—is only increased because of the smaller tear volume. VEGF in the tears of patients with SSc decreased by 20%, which can be explained also by the decreased tear secretion of patients [36].

The question why contrary to our expectations VEGF levels are not higher in patients with SSc than in the healthy group needs further investigation.

2.6.2. Membrane array and multiplex bead analysis of tear cytokines in SSc

Based on our cytokine array results, nine cytokines and chemokines had significantly higher levels in tears of patients with SSc. This screening method was performed for the assortment of 102 cytokines, selecting the most relevant ones in the pathogenesis of SSc for further experiments. All molecules which appeared to be significantly higher in tears of patients are molecular players of the immune responses and inflammatory processes, which confirms the presence of ocular surface inflammation in patients with SSc possibly as a consequence of DES [36].

CHI3L1, a protein which takes part in the processes of inflammation and tissue remodeling, has not been previously described in relation to the pathomechanism of SSc. We have found elevated levels of CHI3L1 in patients with SSc. This result correlates well with the fact that inflammation and tissue injury caused by hypoxia and oxidative stress are always present in the course of SSc.

In fact, different pathways may lead to vascular dysfunction processes in SSc, such as direct vascular damage or pro-inflammatory responses. Studies in different diseases have shown functional links between activated complement molecules and these pathways. CFD, a serine protease, also known as adipsin, plays a key role in these processes [37, 38]. CFD is the rate-limiting enzyme in the activation cascade of the alternative pathway, and its level in the blood is quite low. Our cytokine array results showed increased CFD levels, which confirm the role of the complement system in the ocular pathology of SSc.

Levels of EGF were also elevated in tear samples of patients with SSc. EGF is a growth factor that stimulates cell growth, proliferation, and differentiation [39]. Elevation of EGF may be explained by the above processes of vasculopathy. The next molecule, which appeared to be higher in patients' tears, is matrix metallopeptidase-9 (MMP-9). As a protease of the MMP family, it is involved in the breakdown of extracellular matrix in normal physiological processes, such as embryonic development, reproduction, angiogenesis, bone development, wound healing, cell migration, as well as in pathological processes, such as intracerebral hemorrhage, arthritis, and metastasis [40–42]. In a study of Kim et al., serum MMP-9 concentrations were found to be elevated in patients with SSc correlating well with skin scores [43]. Their results suggest that increased MMP-9 concentrations may be due to their overproduction by dermal

fibroblasts and also that the enhanced production of MMP-9 may contribute to fibrogenic remodeling during the progression of skin sclerosis in SSc. Our results of tear cytokine array are parallel with the finding that MMP-9 is increased in the course of SSc.

In a previous study, expression of antiangiogenic chemokines and their receptors were determined in the sera and skin of patients with SSc [44]. Based on their results, MIG and its receptor are elevated in serum and highly expressed in the skin of patients with SSc. We have also found increased levels of MIG in tear samples of patients, which confirm the fact that dysregulated angiogenesis is an important feature in the pathomechanism of SSc. The next protein that appeared to be higher is VDBP, a member of the albumin gene family. VDBP is a multifunctional protein found in plasma, ascitic and cerebrospinal fluid and on the surface of many cell types. It binds to vitamin D and its plasma metabolites and transports them to target tissues [45]. Others have measured significant quantities of VDBP-actin complexes in the plasma following injury [46]. The presence of tissue injury is likely to be the explanation of our results, namely, the elevated levels of VDBP in the tears of patients with SSc.

Based on our results of multiplex bead assay, the three molecules that showed significant differences in tears of patients and controls were IP-10, MCP-1, and CRP. Previous studies have already demonstrated elevated levels of these markers in the sera of patients with SSc.

General markers of inflammation, such as CRP, are expected to be higher in a disease like SSc. In earlier trials, CRP appeared to be elevated in the sera of patients with SSc and was associated with poor survival. Therefore, it may be a useful indicator of disease activity and severity in SSc [47, 48].

Another inflammatory chemokine, IP-10, also called CXCL-10, has often been investigated in SSc studies [44, 49, 50]. IP-10 has an angiostatic function as it suppresses neovascularization; furthermore, it is involved in immune regulation [51].

Recent reports have shown that the serum and/or the tissue expressions of IP-10 are increased in various bacterial, viral, fungal, and protozoal infections [52] and also in autoimmune diseases like rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis, autoimmune thyroid diseases, type 1 diabetes mellitus, Addison's disease, and SSc [50, 53–55]. CXCL10 is secreted by CD4+, CD8+, natural killer and natural killer T cells and is dependent on interferon-γ. CXCL10 can also be secreted by several other cell types, including endothelial cells, fibroblasts, keratinocytes, thyrocytes, preadipocytes, etc. Detecting a high level of CXCL10 in peripheral fluids is therefore a marker of host immune response [48], which correlates well with our results of cytokine bead assay measurements.

Finally, MCP-1, which is a key participant of the fibrotic processes in SSc, also appeared to be higher in patients' tears. MCP-1, which recruits monocytes, memory T cells, and dendritic cells to the sites of inflammation, is produced by either tissue injury or infection [56]. It is known as one of the most pathogenic chemokines during the development of inflammation and fibrosis in SSc [57]. MCP-1 is not only a chemoattractant molecule for monocytes and T cells, but it also induces Th2 cell polarization and stimulates collagen production by fibroblasts [58]. Hasegawa et al. have previously shown that serum MCP-1 levels are elevated when the skin and lung are affected in patients with SSc [59]. It has also been reported that

cultured dermal fibroblasts from patients with SSc show augmented expressions of MCP-1 mRNA and protein [60].

Of the last three molecules, IP-10 and MCP-1 are the ones whose molecular characteristics single them out as potential candidates for therapies against the pathological consequences of diseases such as SSc.

2.7. Novel findings

1. After reviewing the literature of tear sampling techniques, we labored the adequate tear sampling methods and collected tears with capillary system from SSc patients in order to investigate VEGF molecule and cytokines.
2. We were the first to demonstrate the presence and concentration of VEGF, an element that plays an important vascular role in the pathogenesis of SSc, with the help of a method that is based on a quantitative sandwich immunoassay technique.
3. By the help of our survey, which is based on a quantitative sandwich immunoassay technique, we were able to verify a 20% reduction in the VEGF concentration in tears of SSc patients compared to healthy controls.
4. We were the first to establish a wide cytokine profile in tears of SSc patients using an array that monitors 102 cytokines simultaneously.
5. Based on our cytokine array results, we revealed that 9 out of the 102 cytokines and chemokines had significantly higher levels in tears of patients with SSc. All of them are molecular players of the immune responses and the inflammatory processes. These findings legitimate the existence of ocular surface inflammations which are quite frequent in patients with SSc. In addition, they are in accordance with former study results regarding the pathomechanism of SSc.
6. By using a highly sensitive and specific multiplex bead assay, we were the first to demonstrate increased levels of IP-10, MCP-1, and CRP in tear samples of patients with SSc. Previous studies have already demonstrated elevated levels of these biomarkers in the sera of these patients; therefore tear analysis is to be raised as a potential means in dealing with diagnostic, prognostic, and maybe even therapeutic challenges of SSc.

2.8. Future plans

Angiogenesis impairment in SSc has been proved by several researchers. A number of serum investigations have been carried out regarding this phenomenon, but there are only scant data concerning tears of SSc patients.

The issue why the VEGF levels are not higher in SSc patients than in the healthy group needs further investigation. Other biochemical methods, like PCR, would be feasible to confirm array results. Furthermore, a longer-term prospective study in a larger population with extension of the ophthalmological examinations is needed to confirm clinical utility.

Our current data depict a group of inflammatory mediators, which may play a significant role in ocular pathology of SSc. Monitoring these factors in the tears of patients with SSc can be a noninvasive alternative to serum investigation. Additionally, in patients with ocular manifestations, such as DES, tear analysis is far more informative; it provides information of the ocular surface; hence it could help us choose the appropriate treatment, in particular artificial tears or anti-inflammatory eye drops [36]. Further studies are needed to understand the signaling pathways regulating pro-inflammatory cytokines, with the aim of developing new interventions against autoimmune diseases mediated by cytokines and chemokines, as well as inventing novel therapeutic possibilities for the ocular manifestations of SSc. New inflammatory mediators are to be searched that might function as excellent candidates for future therapeutic targets in SSc with ocular manifestations.

Acknowledgements

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Nomenclatures

AD	Aqueous deficient
BCA	Bicinchoninic acid
CD	Cluster of differentiation
CFD	Complement factor D
CHI3L1	Chitinase-3-like protein 1
CRP	C-reactive protein
CXCL	Chemokine (C-X-C motif) ligand
DES	Dry eye syndrome
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
IFN	Interferon
IL	Interleukin
ILD	Interstitial lung disease
IP-10	Interferon gamma-induced protein-10
MC	Microcapillary tubes
MCP	Monocyte chemoattractant protein
MGD	Meibomian gland dysfunction
MIG	Monokine induced by gamma interferon
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
NST	Nonstimulated tear

PCR	Polymerase chain reaction
PTF	Precorneal tear filmRNARibonucleic acid
RNP	Ribonucleoprotein
sIgA	Secretory immunoglobulin A
SSc	Systemic sclerosis
ST	Stimulated tear
STS	Schirmer test strip
TGF-β	Transforming growth factor-beta
TNF-α	Tumor necrosis factor-alpha
TRIM	Tripartite motif
VDBP	Vitamin D binding protein
VEGF	Vascular endothelial growth factor
WO	Washout

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