Mini Review

Inflammation and pathogenic fibrosis in human ocular chronic graft versus host disease

Yoko Ogawa\(^1,2,\ast\), Shigeto Shimmura\(^1\), Masataka Kuwana\(^3\), Kazuto Yamazaki\(^4\), Yutaka Kawakami\(^2\), and Kazuo Tsubota\(^1\)

\(^1\)Department of Ophthalmology, \(^2\)Division of Cellular Signaling, Institute for Advanced Medical Research, \(^3\)Department of Internal Medicine, \(^4\)Department of Pathology, Keio University, School of Medicine, Tokyo, Japan

Uncontrolled fibrosis due to the excessive accumulation of extracellular matrix proteins plays a primary role in the lacrimal gland dysfunction of patients with ocular chronic graft-versus-host disease (GVHD). To investigate the pathogenesis of this disease, lacrimal gland biopsies obtained from patients with chronic GVHD were analyzed and compared with those from Sjogren’s syndrome (SS) patients, as controls. Increased numbers of CD34\(^+\) fibroblasts and excessive fibrosis in the affected area was prominent, indicating a significant role for stromal fibroblasts in ocular chronic GVHD. The periductal area was the primary site for T-cell and fibroblast activation. A subset of fibroblasts that had accumulated in the affected area expressed HLA-class II and costimulatory molecules; such fibroblasts were not observed in SS lacrimal glands. In addition, donor fibroblast chimerism, which may also contribute to the pathogenic processes of this disease, were detected in the pathogenic fibrotic area. Moreover, T cells interacted with the lacrimal gland myoepithelia, which become HSP47-expressing cells under inflammatory stress. This mini-review will focus on recent researches on the immune response and pathogenic fibrosis in human ocular chronic GVHD, in which we examined the lacrimal gland as one of the targeted exocrine glands. These findings may help elucidate the pathogenesis of lacrimal gland as well as systemic chronic GVHD fibrosis and facilitate the development of novel anti-fibrotic interventions.

Rec.1/31/2008, Acc.10/17/2008, pp529-536

\(^\ast\) Correspondence should be addressed to:
Yoko Ogawa, MD, Keio University School of Medicine, 35 Shinanomachi, Shinjuku, Tokyo 160-8582, Japan. Phone: +81-3-5363-3778, Fax: +81-3-5362-9259, e-mail: yoko@sc.itc.keio.ac.jp

**Key words** bone marrow, dry eye, fibroblast, graft-versus-host disease, pathogenic fibrosis

Chronic graft-versus-host disease (GVHD) is a serious immunological complication that can follow allogeneic hematopoietic stem cell transplantation (HSCT). In long-term survivors after HSCT, chronic GVHD can lead to debilitating conditions involving numerous organs, including the lacrimal gland\(^1\). The main histologic features of chronic GVHD are widespread tissue atrophy and fibrosis with lymphocytic infiltration; however, the pathophysiology of this disease is less understood.
Dry eye is a well known major complication in patients who develop chronic GVHD after HSCT, and it can be associated with potential damage to the ocular surface, including the lacrimal gland and conjunctiva. In a long-term prospective follow-up study, we found that in clinical settings, half the patients who underwent allogeneic HSCT developed dry eye that quickly became severe in most cases.

We have been studying the pathogenesis of chronic GVHD by examining the involvement of the lacrimal gland, one of the preferentially targeted organs in this disease. Pathogenic fibrosis due to the excessive accumulation of extracellular matrix proteins plays a primary role in the lacrimal gland dysfunction of patients with ocular chronic GVHD. We also found the periductal area as the primary site for T-cell and fibroblast activation based on the detection of CD154 on T cells, an activation marker of T cells, and the elevated expression of heat shock protein 47 on fibroblasts, a marker of collagen-secreting cells. Fibroblasts that had accumulated in the affected area expressed HLA-class II and costimulatory molecules as full component necessary for antigen presentation; such fibroblasts were not observed in SS lacrimal glands, suggesting that a unique subpopulation of fibroblasts contributes to the pathogenic fibrosis in lacrimal gland in patients with chronic GVHD. By detecting mismatched genetic markers in tissue specimens and primary fibroblast cultures, we determined that nearly half of the CD34+ fibroblasts at the site of pathogenic fibrosis were of donor origin. Moreover, T cells interacted with the lacrimal gland myoepithelium, which become HSP47-expressing cells under inflammatory stress, facilitating the production of collagen bundles by the myoepithelia. This mini-review will focus on the lacrimal gland immune and inflammatory response and fibrosis, a characteristic feature of the lacrimal gland in patients with chronic GVHD.

Stromal fibroblasts play a major role in the rapidly progressive dry eye in chronic GVHD

Chronic GVHD is the main cause of morbidity and mortality in HSCT recipients, and thus an important barrier to the success of HSCT. Because of the increasing number of long-term survivors who have received allogeneic HSCT, dry eye has a significant impact on more of these patients’ quality of life, and can potentially cause significant visual loss, and even blindness. However, the pathogenic process of the dry eye associated with chronic GVHD remains largely unknown, and little attention has been paid to the ocular complications in chronic GVHD.

We first performed a comprehensive analysis to elucidate the histopathologic features of the lacrimal gland in chronic GVHD after allogeneic HSCT. We analyzed the lacrimal gland specimens from five patients who had presented with dry eye as part of their symptoms of chronic GVHD, by immunohistochemistry and transmission electron microscopy. Lacrimal gland specimens from five patients with Sjogren’s syndrome (SS) were used as controls.

The main histologic findings in the affected lacrimal gland were marked fibrosis of the interstitium and a pronounced increase in the number of CD34+ fibroblasts, with mild lymphocytic infiltration (Fig. A, B). In patients with chronic GVHD, lymphocytes, mainly consisting of T cells, were found primarily in the periductal areas of the lacrimal gland, while in SS patients, B cells were the dominant infiltrating cells in the lacrimal gland, and were found in the acinar areas. These findings in the chronic GVHD patients were more prominent in severe than mild dry eye. Electron microscopy of the chronic GVHD lacrimal gland revealed stromal fibroblasts attached to various inflammatory cells, especially T cells with clustered dense bodies, via primitive contacts. In addition, well-developed rough endoplasmic reticulum in the fibroblasts and newly synthesized collagen fibrils in the extracellular matrix were observed, indicating that extracellular matrix components were being actively produced. We also observed that the blood vessels, ducts, and lobules in the chronic GVHD lacrimal gland had multilayered and thickened basal laminae, which were only rarely seen in the lacrimal gland of SS patients. Thus, our observations showed substantial differences between the lacrimal gland histopathology of patients with chronic GVHD and SS patients. Moreover, our findings indicated that the pathogenic process of chronic GVHD in the lacrimal gland may involve the active production of excessive extracellular matrix components by stromal fibroblasts.

Clinically, we found that the severity of dry eye correlated with the extent of fibrotic changes, rather than with the amount of lymphocytic infiltration, indicating that excessive extracellular matrix accumulation is the major contributor to lacrimal gland dysfunction.

The periductal area is the primary site of T-cell activation in chronic GVHD lacrimal glands

It is widely accepted that donor T cells are responsible for the pathogenesis of GVHD. Acute GVHD is thought to result from an alloimmune response of donor T cells to the recipient cells and tissues, followed by dysregulated cytokine production and
additional effector-cell recruitment\textsuperscript{3,14}. However, the pathogenic process of chronic GVHD is still unknown. Therefore, to investigate the immune processes associated with chronic GVHD, we examined the expression of surface molecules associated with T-cell activation in the lacrimal gland of patients with this disease\textsuperscript{6}. Lacrimal gland biopsies obtained from nine patients with chronic GVHD and five with SS were subjected to immunohistochemical analysis with antibodies against CD4, CD8, CD34, CD40, CD54, CD80, CD86, CD154, and HLA-DR. The regions of interest were then analyzed by transmission electron microscopy. CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells were detected primarily in the periductal areas of the lacrimal glands of chronic GVHD patients, but were distributed throughout the acinar areas in those of SS patients. In the chronic GVHD patients, a subpopulation of these periductal CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells expressed the activation marker CD154. Furthermore, colocalized with these CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells were mononuclear infiltrates and stromal fibroblasts expressing all the surface molecules necessary for antigen presentation, including HLA-DR, CD54, CD40, CD80, and CD86\textsuperscript{6}. The electron microscopic analysis revealed activated fibroblasts whose processes enveloped with lymphocytes and macrophages. In addition, more CD8\textsuperscript{+} T cells were observed in the glandular epithelia of chronic GVHD patients than in that of SS patients. The intra-epithelial T cells were attached to myoepithelial cells with several primitive contacts, and dead cells were also co-localized with these T cells\textsuperscript{6}.

These observations — the accumulation of CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells, CD154\textsuperscript{+} activated T cells, and mononuclear cells and stromal fibroblasts expressing HLA-DR, CD54, and costimulatory molecules, around the glandular ducts — identified the periductal area as the primary site for T-cell activation in HSCT recipients with lacrimal gland chronic GVHD. Interestingly, the periductal area is also where increased stromal fibroblasts, T cells and fibroblasts interaction and excessive fibrosis occur in chronic GVHD patients\textsuperscript{6}. Our results support a mechanism for chronic GVHD in which CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells in the lacrimal gland are activated primarily in the periductal area by antigenic stimulation from potent antigen-presenting cells such as macrophages and stromal fibroblasts, and the activated T cells then exert effector functions, including cytotoxic effects, on the lacrimal gland epithelial cells. The identification of the T cells that had infiltrated the ductal epithelia in chronic GVHD as activated CD8\textsuperscript{+} cytotoxic T cells indicated that the destruction of the ductal epithelium was due to T-cell invasion. It is likely that the CD8\textsuperscript{+} T cells target the lacrimal gland myoepithelium adjacent to the basal lamina.

The finding that the stromal fibroblasts in the lacrimal gland of chronic GVHD patients express human leukocyte antigen class II and costimulatory molecules such as CD40, CD54, CD80, and CD86, while SS fibroblasts do not, suggests that the fibroblasts that accumulate at the pathogenic fibrosis site in patients with chronic GVHD are a unique and unusual subpopulation.

Further investigation aimed at identifying the target antigens recognized by the infiltrating donor T cells and the functional properties of the stromal fibroblasts should elucidate the mechanism of dry eye pathogenesis in patients with chronic GVHD.

**Donor fibroblast chimerism in the pathogenic fibrotic lesions of lacrimal gland chronic GVHD**

Recent evidence suggests that the engraftment of non-hematopoietic cells occurs in various organs of HSCT recipients\textsuperscript{15-19}. In addition, recent findings in animal models indicate that a significant proportion of the mesenchymal cells involved in tissue injury may be derived from the bone marrow or from circulating fibrocytes\textsuperscript{20,21}. These reports and our previous findings on the unique phenotype of fibroblasts in chronic GVHD lesions led us to hypothesize that a subset of the fibroblasts that accumulated at chronic GVHD lesions originated from the transplanted donor cells. To test this hypothesis, we next investigated the origin of the CD34\textsuperscript{+} fibroblasts that accumulate in the chronic GVHD lacrimal gland, by detecting mismatched genetic markers in tissue specimens and in primary fibroblast cultures\textsuperscript{6}.

Lacrimal gland biopsies were obtained from nine patients with chronic GVHD. Of these, seven were females who had received a transplant from male donors. In the samples from these patients, male-specific sequences detected by fluorescein in situ hybridization (FISH) and in situ hybridization (ISH) could be used as markers for the donor cells. The lacrimal gland biopsies were used to generate primary fibroblast cultures, which were examined for mismatched genetic markers between recipients and donors. To confirm whether CD34\textsuperscript{+} cells accumulated in the ocular chronic GVHD were fibroblasts, lacrimal gland and conjunctival tissue was subjected to CD34 (Fig.1C) and factor VIII (Fig.1D) in consecutive sections and double-staining for CD34 in combination with CD90 (Fig.1E) or CD45 (Fig.1F). In consecutive sections, CD34-positive fibroblasts in the subconjunctival stroma were confirmed to be negative for factor VIII (Fig.1D). In addition, CD34 positive cells forming circular lesions corresponding to capillary walls were positive for factor VIII. The majority of CD34\textsuperscript{+} cells in the interstitium co-expressed a fibroblast marker CD90\textsuperscript{25}, but only half of CD90\textsuperscript{+} fibroblasts expressed
Fig 1  Typical histologic findings of the lacrimal gland in patients with chronic GVHD
(A) Hematoxylin and eosin staining of a section from chronic GVHD patient #14 (original magnification x100). D, duct. (B) CD34 immunostaining of the consecutive section of A (original magnification x100). A high-magnification view is shown in the inset (original magnification x400). (C,D) In consecutive sections, CD34-positive fibroblasts in the subconjunctival stroma (Fig.C) were confirmed to be negative for factor VIII (Fig.D). CD34 positive cells forming circular lesions corresponding to capillary walls were positive for factor VIII (Fig.D). (E) Immunofluorescent double-staining of CD34 (green) and CD90 (red) on a lacrimal gland section from chronic GVHD patient #17 (original magnification, x630). Nuclei were counterstained with TO-PRO-3 (blue). (F) Immunofluorescence double-staining of CD34 (green) and CD45 (red) on a lacrimal gland section from chronic GVHD patient #17 (original magnification, x630). Nuclei were counterstained with TO-PRO-3 (blue). (Reprinted from reference 9, with permission from Investigative Ophthalmology and Visual Science)

CD34 (Fig.1E), indicating that there are CD34+ and CD34- fibroblasts in the lacrimal gland from chronic GVHD patients. In contrast, none of CD34+ cells simultaneously expressed a hematopoietic marker CD45, while many CD45+ round cells were located around spindle-shaped CD34+ cells (Fig.1E). Taken together, these findings indicate that the majority of CD34+ spindle-shaped cells accumulated in the interstitium of ocular cGVHD lesion are non-hematopoietic fibroblasts.

Although both CD34+ and CD34- fibroblasts accumulate in the lacrimal gland from patients with chronic GVHD, we had to use CD34 as a marker for fibroblasts because there is no reliable
fibroblast marker that can be used in the tissue sections. This limitation should be considered upon interpreting our findings.

In the samples obtained from the seven female patients who had received a male transplant, FISH for the Y-chromosome revealed that 13.4-26.7% of the CD34+ fibroblasts that had accumulated in the fibrotic lesions were derived from the donor origin (Fig.2A,B, Table 1). We also noted Y-FISH+ donor-derived cells negative for CD34 in the interstitium and in the epithelia of acini and ducts. The majority of these cells were likely to be lymphocytes (Fig.2C,D, arrowheads), but some may be CD34 fibroblasts, and epithelial cells. Male-specific mRNA was also detected in the lacrimal gland fibroblasts, by ISH.

Next, primary fibroblast cultures were generated from the lacrimal gland of four of these chronic GVHD patients and further examined for mismatched genetic markers between recipients and donors (Table 1). Y-chromosome sequence detection and donor-specific microsatellite genetic markers again indicated the presence of fibroblasts of donor origin. Theoretically, this method should allow us to detect donor-derived cells in cultured fibroblasts irrespective of the combination of donor/recipient genders. These findings indicate a chimeric status of the accumulated CD34+ fibroblasts in the lacrimal gland of chronic GVHD patients (Table 1). Fibroblasts originating from circulating donor-derived precursors may contribute to the excessive fibrosis found in patients with lacrimal gland chronic GVHD, and possibly also to the development of the disease. These unique fibroblasts might release an unusual pattern of cytokines that function to amplify the lacrimal gland chronic GVHD microenvironment.

These observations have important implications regarding the pathogenesis of chronic GVHD. It was originally thought that chronic GVHD was a later phase of acute GVHD, which arises from allo-recognition by donor T cells, but other studies suggest that an autoimmune-like process induced by dysfunctional immunologic recovery also plays a role. In addition, the apparent acceleration of the fibrotic process and the resultant excessive fibrosis leads to functional impairment in various organs, including the lacrimal gland. Our findings suggest that donor-derived fibroblasts may play a role in this pathogenic fibrosis. It has been proposed that, in HSCT recipients, tissue injury from acute GVHD, the conditioning regimen, or some other source facilitates the homing of circulating stem cells or precursors and their differentiation into various tissues, in response to signals in the local environment. Under such a scenario, the persistently fibrotic environment in chronic inflammatory lesions might promote the recruitment and mobilization of donor-derived fibroblast precursors.

Our study was the first to demonstrate the presence of bone marrow-derived fibroblasts in the pathogenic fibrosis of chronic GVHD. This finding led us to hypothesize that bone marrow-derived fibroblasts contribute to the pathogenesis of human fibrotic diseases, such as scleroderma, Stevens Johnson syndrome, and ocular cicatricial pemphigoid. Although no effective treatment has yet been found for lacrimal gland chronic GVHD fibrosis, strategies that inhibit the recruitment of fibroblast precursors into the affected lesions to slow the fibrotic process represent a novel therapeutic approach for lacrimal gland chronic GVHD as well as systemic GVHD.
Role of heat shock protein 47 in lacrimal gland pathology

Heat-shock protein 47 (HSP47) is a molecular chaperon that assists the processing of collagen type I to type IV, which is involved in the molecular maturation of collagen, and has been shown to have a fibrogenic role in various human and murine fibrotic diseases\(^3\). We recently investigated the role of HSP47 in the pathogenesis of lacrimal gland chronic GVHD\(^9\).

First, we examined the expression of HSP47, Ki67, collagen types I and III, and \(\alpha\)-smooth muscle actin (\(\alpha\)-SMA) in tissue sections and in primary cultures of fibroblasts obtained from the lacrimal glands and conjunctiva of eight chronic GVHD and eight SS patients. We observed that HSP47 and Ki67 were co-expressed in the matrix-producing fibroblasts, especially in the lacrimal gland, supporting our previous assertion that the periductal area is the primary site for fibroblast activation and the subsequent lacrimal gland fibrosis in chronic GVHD patients\(^9\).

Sections of lacrimal gland from chronic GVHD patients showed a markedly greater expression of HSP47 in the fibroblasts around the medium-sized ducts compared with the samples from SS patients. The elevated HSP47 expression in chronic GVHD patients was mostly detected in Ki67-positive fibroblasts, and it was associated with a high accumulation of types I and III collagen in and around the fibrotic areas. Primary fibroblast cultures from the lacrimal gland of chronic GVHD patients showed greater HSP47 mRNA expression than did those from SS patients, as determined by RT-PCR (\(p<0.05\)). In contrast, the fibroblasts from SS lacrimal glands showed higher \(\alpha\)-SMA than did those from chronic GVHD biopsies at both the mRNA and protein levels, and tissue sections showed more lacrimal gland fibroblasts that were positive for \(\alpha\)-SMA in the SS than in the chronic GVHD samples (\(p<0.01\)).

The increased HSP47 expression in chronic GVHD may promote excessive collagen assembly in and near the periductal areas, where fibroblasts and T cells are mostly in an active state. Our finding that there were fewer \(\alpha\)-SMA-positive fibroblasts in the lacrimal gland of chronic GVHD patients compared with SS patients suggested that the periductal fibroblasts in chronic GVHD were largely incapable of myofioblastic transformation, and were therefore of donor origin, supporting the possibility that donor-derived fibroblasts contribute to the pathogenic process of lacrimal gland chronic GVHD\(^9\).

In addition, we found HSP47 expression in the myoepithelium of ducts and acini in chronic GVHD lacrimal glands. It was recently reported that inflammatory mediators that are produced in response to injury cause the epithelial-mesenchymal transition (EMT)\(^2\). The HSP47 expression in the epithelium could therefore be an early sign of EMT. Our finding that HSP47 was expressed by myoepithelial cells and our observation by electron microscopy that collagen fibrils were adjacent to the myoepithelial cells suggested that myoepithelial cells secrete collagens. These results indicate that the myoepithelium may also contribute to the production of excessive extracellular matrix in chronic GVHD lacrimal glands\(^9\).

Our reports support the following series of events occurring in ocular chronic GVHD involving the lacrimal gland and conjunctival fibrosis (Fig. 3). First, breakdown of the blood vessel basal laminae results in the migration of donor-derived fibroblasts and T cells into the ocular tissues, perhaps due to homing signals released as part of an alloimmune response. These T cells are then activated and migrate into the periductal areas where

![Fig.3 Schematic representation of various events of lacrimal gland cGVHD fibrosis](image-url)
they interact with the lacrimal gland epithelia to promote activation and transition from the myoepithelial to the mesenchymal phenotype, which become HSP47-expressing cells under inflammatory stress\(^{25}\), facilitating the production of collagen bundles by the myoepithelia. At the same time, donor-derived fibroblasts become activated and proliferate, induced by cytokines released from inflammatory cells. These activated fibroblasts further express high levels of HSP47, which exacerbates the production of excessive extracellular matrix. The abundant extracellular matrix contributes to interstitial fibrosis, with the end effect being impaired lacrimal gland function. Donor-derived fibroblasts are incapable of transforming into myofibroblasts, and may have a prolonged lifespan, during which they continue to produce excessive extracellular matrix.

Previous reports have suggested that the pathophysiology of chronic GVHD may be due to autoreactive T cells that have escaped negative selection in the thymus, which may have been damaged by preconditioning or preceeding acute GVHD\(^{26,27}\). Donor fibroblasts expressing HLA-DR, and co-stimulatory molecules may act as antigen presenting cells that trigger chronic GVHD. Migrating donor fibroblasts may interact and collaborate with recipient derived T cells and fibroblasts to contribute to the development of chronic GVHD (Fig.3).

Fibroblasts represent a heterogeneous population and are thought to be derived from three different sources in lacrimal gland chronic GVHD fibrosis: donor-derived fibroblasts, preexisting stromal fibroblasts, and epithelia-derived fibroblasts via EMT. Studies have shown that during kidney fibrosis approximately 15% of the fibroblasts originate from circulating precursors, including those from bone marrow, and 36% arise via EMT\(^{28}\). Further studies need to be performed to clarify the main source of the fibroblasts contributing to the pathogenesis of lacrimal gland chronic GVHD.

In conclusion, the suppression of migrated or activated fibroblasts early in the course of lacrimal gland chronic GVHD could lead to the prevention of progressive dry eye as well as systemic chronic GVHD fibrosis. Further studies investigating the inflammatory and fibrotic processes, especially on the origin of the fibroblasts that contribute to the pathogenic fibrosis in chronic GVHD would be useful for clarifying its pathogenesis as well as for the development of focused therapeutic interventions for this inflammatory and fibrotic disease.

Acknowledgements

We thank Drs Kaori Kameyama, Mohammed S Razzaque, and Shinichiro Okamoto for their collaboration and critical comments regarding these studies. We also thank Toshihiro Nagai for Expert technical assistance. This study was supported by grants from the Japanese Ministry of Education, Science, Sports and Culture No 20592058.

References


