Graft-versus-host disease (GVHD) is the major cause of morbidity and mortality among patients undergoing allogeneic hematopoietic stem cell transplantation. Although animal models have been clearly established for the study of skin, liver, and gut, currently there is no equivalent experimental model for analyzing ocular involvement, which is rather common, especially among patients diagnosed with chronic GVHD. In the current study we have developed a murine model of ocular GVHD and, for the first time, we describe the histopathologic features involving cornea and limbus, which could play a role in the physiopathology of the disease at the ocular level. Our results represent a major finding that allows us to define a model for evaluating new therapeutic strategies for treating ocular GVHD prior to their use in clinical setting.

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INTRODUCTION

Graft-versus-host disease (GVHD) is the major cause of morbidity and mortality among patients undergoing allogeneic hematopoietic stem cell transplantation (HSCT) [1-3]. Among target organs, ocular involvement has been reported in up to 60% of patients [4,5]. Although it mostly appears in the chronic GVHD (cGVHD) setting, it can also be a manifestation of acute GVHD (aGVHD) [2,6]. Ocular GVHD affects all layers of the eye, but the most commonly affected is the ocular surface [7]. The ophthalmic clinical features include keratoconjunctivitis sicca, punctate keratopathy, persistent epithelial defects, corneal keratinization, ulceration, and perforation [8]. Posterior segment manifestations are less frequent and include microvascular retinopathy, intraretinal and vitreous hemorrhage, central serous chorioretinopathy, and scleritis. GVHD also affects the eyelids, causing dermatitis, lagophthalmos, ectropion, poliosis, madarosis, and vitiligo [8-11]. Dry eye is the main ophthalmologic complication among patients with cGVHD [12], and although several treatments have been used to minimize its symptoms, therapeutic options are limited [13-15]. Furthermore, although ocular involvement does not affect the outcome of the patients with respect to mortality, different studies on quality of life recognize this complication as being a major concern [2,3,5,15,16].

Despite its impact on the morbidity associated with transplant, the histopathology of ocular involvement is not well understood, because most studies have focused on clinical manifestations. A few reports have examined ocular histopathology in GVHD, describing the histologic features in the conjunctiva and lachrymal glands [14,17,18]. Nevertheless, the histopathology of the cornea and limbus continues to lack thorough examination [7,8,19], mainly because there are no animal models that allow us to determine the involvement of these layers with greater accuracy.

The current study establishes a murine model of GVHD and describes for the first time the histopathologic features involving cornea and limbus that explain many of the clinical characteristics of this syndrome.

MATERIALS AND METHODS

Animals

All animal protocols were approved by the University of Salamanca Animal Care and Use Committee.
Female BALB/c (H2d) and male C57BL/6 (H2b) mice were purchased from Charles River Laboratory, France. Animals were kept in specific pathogen-free conditions. Mice were between 8 and 12 weeks of age at the beginning of the experiments. Donor mice were killed by cervical dislocation, and their bone marrow (BM) and spleen from C57BL/6 were then harvested by standard techniques. Spleen cell preparations were prepared by gently crushing the tissues to release the cells. Preparations were filtered to remove debris and washed twice in phosphate-buffered saline (PBS) before injection.

BALB/c (H2d) mice were used as recipients in the GVHD model systems. Recipient mice received total body irradiation (TBI) (850 cGy divided into 2 fractions) from a Cs source. Irradiation was followed by the infusion of $5 \times 10^6$ C57BL/6 allogeneic donor BM cells intravenously with or without splenocytes ($5-10 \times 10^6$ cells intravenously) as a source of allogeneic T cells. Mice were monitored and weighed twice a week. All moribund mice were humanely killed.

The degree of systemic GVHD was assessed by a standard scoring system from 0 to 2, which incorporates 5 clinical traits: weight loss, posture (hunching), activity, fur texture, and skin integrity [20]. Three clinical features of the eyes were also monitored: periocular fur, eyelid margin, and blepharospasm. Each characteristic was scored between 0 (no loss of periorcular fur, no crusting of the eyelid margin/erythematous lids, no blepharospasm), 1 (1 feature of the following: loss of periorcular fur, crusting of the eyelid margin/erythematous lids, blepharospasm), and 2 (2 or 3 of the previously mentioned features). Transplanted mice were ear-punched, and individual weights were recorded on day 0 and weekly thereafter. At the time of analysis, mice from coded cages were evaluated and graded for each criterion.

Mice were killed 2 months after allogeneic HSCT by CO₂ asphyxiation followed by cervical dislocation. Afterward we removed the right and left eyes, lachrymal glands, and eyelids. We also analyzed portions of skin, liver, and intestine. The enucleated eyes, eyelids, and lachrymal glands were fixed in neutral buffered formalin and embedded in paraffin. The eyes were then sectioned sagitally through the optic nerve and processed for hematoxylin-eosin staining.

**Pathological Examination**

Slides were coded and examined by a trained pathologist. Eye specimens were routinely processed and embedded in paraffin and serially sectioned. Slides were then deparaffinized and rehydrated. After gentle washing, the slides were stained with hematoxylin for 5 minutes, washed again, and stained with eosin for 1 minute. Samples were then dehydrated in 95% ethanol to remove excess eosin. Finally, slides were dried in xylene overnight.

The pathologist analyzed the features of GVHD present in gut, liver, and skin as well as those most commonly reported in the conjunctiva and lachrymal gland of the human eye [19,21,22]. Apoptosis was ascertained by assessing the presence of chromatin clumping, nuclear pyknosis, or nuclear debris and was confirmed using caspase 3 (Novocastra™ Lyophilized Mouse Monoclonal Antibody CPP32).

**RESULTS AND DISCUSSION**

All mice receiving donor splenocytes exhibited clinical features $\geq 1$ of GVHD 30-60 days after allogeneic BM transplantation: weight loss, hunched posture, abnormal fur texture, poor skin integrity, and decreased activity. External ocular findings included (1) loss of periorcular fur, (2) crusting of the eyelid margin or erythematous lids, and (3) blepharospasm. Control mice receiving donor BM cells intravenously without splenocytes were also sacrificed the same day posttransplant as those developing GVHD and were used as controls.

Histopathologic examination of gut, liver, and skin showed signs of GVHD. Macroscopic examination of the enucleated eyes showed corneal ulcers (Figure 1B). Pathologic examination revealed the presence of histologic features of GVHD. Thus, eyelid biopsies showed a lymphocytic infiltrate at the dermal and epidermal junction with vacuolization of epidermal cells. Satelliteosis, that is, lymphocytes surrounding apoptotic epidermal keratinocytes, were observed. We also found apoptosis among basilar keratinocytes. All these data provided evidence of GVHD-induced dermatitis.

Lachrymal gland specimens showed eosinophilic focal infiltration in the interlobular area in 8 of 10 sections analyzed, whereas there were no mononuclear cells in the acinar area. A number of eosinophils appeared to infiltrate the ductal epithelia, and apoptotic bodies were also observed. Finally, fibrosis was observed surrounding lachrymal gland ducts.

The corneal epithelium was atrophic and showed vacuolization of the epithelial cells of the basal and medium layers of the cornea. Two or more apoptotic cells with infiltrating leukocytes were also observed in corneal epithelium in at least 8 of 10 sections analyzed. Furthermore, diffuse stromal edema with neovascularization and inflammatory infiltrate was observed. No significant abnormalities were observed in the endothelium (Figure 1).

In bulbar and tarsal conjunctiva we observed dyskeratosis with loss of conjunctival epithelium, lymphocyte exocytosis, satellitosis, and epithelial cell necrosis, with apoptotic bodies in the epithelium in 8 of 8 samples analyzed. Subepithelial microvesicles and mononuclear cells infiltrating the substantia propria were
also observed in the bulbar and tarsal conjunctiva (Figure 2).

Notably, limbus epithelium showed the same histologic features as those observed in corneal epithelium, with satellitosis, vacuolization of cells, and presence of apoptotic bodies as suggested by the presence of chromatin clumpings and nuclear pyknosis in at least 8 out of 10 biopsies analyzed. In addition, edema of corneal stroma was also observed (Figure 3).

None of the findings were found, neither in the external nor in the histopathologic examination among control mice receiving TBI plus BM cells. Accordingly, all these features are specific for GVHD and not for stress, TBI, or other nonspecific causes.

GVHD is a complex immunologic process that involves several organs. Ocular manifestations are found in the majority of patients and have been reported in up to 80% of patients with cGVHD [7]. Although less frequent, ocular damage has also been described in the aGVHD setting.

Although the transplant model used in the current study is widely used, ocular involvement has not been previously reported. Accordingly, our results represent a major finding that allows a model of ocular GVHD to be established. In this regard, although histopathologic findings have already been described in other target organs, this is not the case for cornea and limbus and, furthermore, an animal model has not been previously

Figure 1. Light microscopic findings of cornea in mice with ocular GVHD. Hematoxylin and eosin staining. Original magnification ×40. (A) Shows cytoplasmic vacuolization in the basal layer of the epithelium (arrow) and stromal edema of the cornea (blue arrow). (B) A high magnification view of A shows a detail of vacuolization cells with apoptotic bodies and satellitosis of the cornea. (C) Epithelial necrosis of the cornea showing apoptotic bodies (arrow) and stromal and epithelial inflammatory infiltrate. (D) Corneal stromal vascularization (arrow), edema, and lymphomononuclear cell infiltrate.

Figure 2. (A) Light microscopic findings of conjunctiva with macroscopic ocular GVHD. Hematoxylin and eosin staining. This view shows an inflammatory infiltration of the conjunctiva and slight lymphomononuclear cell infiltrate of the sclera (blue arrow). A focal apoptotic bodies formations are present at the corneal epithelium, (shown as chromatin clumpings and nuclear pyknosis) (black arrow) (original magnification, ×100). (B) Caspase 3 staining confirming the presence of apoptotic bodies in the conjunctiva.
standardized that could be most useful for evaluating new therapeutic strategies prior to their use in the clinical setting. Furthermore, the current study allowed us to analyze more accurately the pathologic findings in ocular GVHD. It has not previously been possible to explore these because of the lack of a clearly established animal model. In fact, several findings have already been reported, especially those affecting lachrymal glands, conjunctiva, eyelids and, to a lesser extent, cornea [15-18]. Nevertheless, in the current study we have shown that ocular GVHD also involves cornea and limbus, which show histopathologic findings similar to those observed in other target organs.

In conclusion, the current study establishes an animal model for the evaluation of ocular GVHD and identifies limbus as a target organ.

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