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Keratocyte Apoptosis and Failure of Corneal Allografts

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Abstract

Background—Murine models of high-risk and low-risk corneal transplantation were used to determine the role of keratocyte apoptosis in the failure of orthotopic allogeneic corneal transplants.

Materials and Methods—Normal (low-risk, low-rejecting) and inflamed/vascularized (high-risk, high-rejecting) BALB/c recipient beds received fully mismatched C57BL/6 corneal allografts. Apoptosis was detected in the corneal stroma at various time points using an in situ terminal deoxynucleotide tranferase-mediated dUTP nick-end labeling assay, and ex vivo via Western analysis for active caspase-3. Apoptosis was also measured in a (donor-type) C57BL/6 keratocyte cell line after stimulation of Fas or via use of various pro-inflammatory cytokines.

Results—Significantly more apoptotic cells were present in the stroma of rapidly rejecting high-risk corneal allografts compared with low-risk grafts. Apoptotic cells were shown to be nearly uniformly CD45− and hence of a non-hematopoietic lineage. Apoptosis, however, was not present in highly inflamed but ungrafted corneas. Apoptosis was induced in keratocytes in vitro by dual stimulation with agonistic Fas mAb and either interleukin-1β or tumor necrosis factor-α.

Conclusion—Apoptosis of resident non-bone marrow-derived fibroblastic cells of the corneal stroma is strongly correlated with the failure of corneal allografts, particularly in the highly inflamed microenvironment of the high-risk allograft.

Keywords
Cornea; Apoptosis; Immune rejection

Orthotopic corneal transplantation is the most common and successful form of solid tissue grafting performed, with nearly 40,000 cases per year in the United States alone, and a 2-year survival rate as high as 90% for uncomplicated first grafts performed in normal, non-vascularized, low-risk (LR) beds (1). However, these results contrast sharply with the fate of corneal grafts placed in high-risk (HR) beds, for which rejection rates can exceed 70% to 90%, even with maximal local and systemic immune suppression. The primary cause of corneal allograft failure is immune-mediated rejection characterized by delayed-type hypersensitivity to donor alloantigens and leukocytic cellular infiltration of the graft site (2). However, the precise mechanisms by which corneal transplants fail, characterized by their loss of transparency, remain elusive.

In the present study, we hypothesized that corneal allograft failure is mediated, at least in part, by apoptosis of resident corneal cells, especially of the differentiated fibroblasts of the corneal...
stroma known as keratocytes. The well-characterized murine model of allogeneic corneal transplantation between fully mismatched C57BL/6 donors and BALB/c recipients was used. BALB/c hosts were either LR recipients, in which no corneal inflammation or vascularity was present at the time of transplantation, or HR recipients, in which intense corneal inflammation with accompanying neovascularization was present at the time of transplantation. Our results strongly suggest that apoptotic death of corneal stromal keratocytes at the tissue level, before clinically detectable graft opacification, is a harbinger of graft failure, especially in HR grafts that are particularly susceptible to rejection.

MATERIALS AND METHODS

Experimental Animals

We purchased 6- to 9-week-old BALB/c and C57BL/6 mice from Taconic Farms (Germantown, NY) or the Jackson Lab (Bar Harbor, ME), or obtained them from the Schepens Eye Research Institute breeding facility. All protocols were approved by the Schepens Eye Research Institute Animal Care and Use Committee, and all animals were treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Cell Culture

Immortalized keratocytes from corneal stroma of C57BL/6 mouse (MK/T-1 cells) were obtained as a generous gift from R.L. Gendron (Memorial University of Newfoundland, St. John’s, Newfoundland, Canada). MK/T-1 cells were grown in low-glucose Dulbecco’s minimum essential medium supplemented with 10% fetal bovine serum (FBS) and 1 mM L-glutamine at 37°C in 5% CO₂. Jurkat cells were grown in Roswell Park Memorial Institute-1640 supplemented with 10% FBS at 37°C in 5% CO₂.

Corneal Transplantation

Orthotopic corneal transplantation was performed across both major histocompatibility complex (MHC) and minor histocompatibility antigenic barriers between normal C57BL/6 (donors) and BALB/c (recipients) mice according to our standard protocol as described previously (3). Briefly, the central 2-mm diameter of the donor cornea was excised and replaced in the corresponding central corneal portion of the recipient (1.5 mm diameter), whose cornea has been removed. The corneal button was secured with eight interrupted 11-0 nylon sutures. HR allografts were performed on recipient corneal beds in which inflammation accompanied by neovascularization had been induced 2 weeks before transplantation, as described previously (4). LR allografts were performed on unaltered-normal recipient corneal beds. Syngeneic grafts were performed between normal C57BL/6 donors and recipients as controls. Each experimental group studied comprised 10 grafted hosts; experiments were repeated at least twice. Graft success and failure were measured by the standard grading scheme for evaluation and scoring of orthotopic corneal transplants (1).

Induction of Non-Transplant–Related Corneal Inflammation

To separate the contribution of (allo) antigen-specific from non-antigen specific inflammation to the tissue response, corneal inflammation was induced without associated transplantation via placement of three intrastromal sutures in the corneal stroma of 8- to 12-week-old male BALB/c mice (MHC class II, Ia⁺) as previously described (5). Anesthetized mice received sutures in the central cornea of the right eye. This model reliably induces innate immune responses with associated vascularization of the cornea within 2 weeks, as previously described (5).
In Situ Terminal Deoxynucleotide Tranferase-Mediated dUTP Nick-End Labeling Assay

To detect apoptosis in the cornea, the terminal deoxynucleotransferase UTP nick-end labeling (TUNEL) assay (ApopTag, Serologicals Corp., Norcross, GA) was performed on cryosections of enucleated eyes according to manufacturer’s instructions. Briefly, 5-μm thick sections of whole eye were mounted on glass slides and fixed in 10% buffered paraformaldehyde for 10 min at room temperature. Slides were then postfixed in a 2:1 mixture of ethanol:acetic acid for 5 min at −20°C. Sections were reacted with a preparation of TdT enzyme for 60 min at 37°C in a humidified chamber. After the reaction was stopped with stop/wash buffer, sections were incubated with fluorescein-conjugated anti-digoxigenin for 30 min at room temperature in a dark humidified chamber. Sections were then mounted with Vectorshield mounting media with di-amidino phenyl indole (DAPI) counterstain (Vector Labs, Burlingame, CA) under glass coverslips. TUNEL-positive cells in the cornea were identified on a Nikon E800 epifluorescence microscope.

CD45 Immunofluorescence

To determine whether bone marrow-derived leukocytes were undergoing apoptosis in the cornea, eye sections were concurrently stained for CD45 (pan-leukocyte marker) and the TUNEL assay. TUNEL assay was performed as described above but interrupted after the TdT reaction was stopped. At this point, sections were blocked with 2% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 30 min, then incubated in 1 μg/mL rat anti-mouse CD45 (BD Pharmingen) for 2 hr. Sections were washed and incubated in 1 μg/mL rhodamine-conjugated goat anti-rat IgG (BD Pharmingen) for 1 hr. All blocking and antibody incubation steps were performed at room temperature in a humidified chamber. After a final wash, the TUNEL assay was completed and sections were mounted with Vectorshield mounting media with DAPI counterstain under glass coverslips.

In Vitro Induction of Apoptosis in Keratocytes

Confluent MK/T-1 cells were treated for 24 hr with the agonistic monoclonal antibody to murine Fas (Jo2; BD Pharmingen) at 10 ng/mL, alone or in combination with 10 ng/mL recombinant murine interferon-γ, recombinant murine interleukin-1β, or recombinant murine tumor necrosis factor-α (R&D Systems). Apoptosis in cultured Jurkat cells (a human T-cell line) was used as a positive control for active caspase-3 expression as they readily undergo apoptosis under in vitro experimental conditions. Confluent Jurkat cells were treated for 24 hr with 1 μg/mL staurosporine (Sigma).

Western Blot Analysis for Active Caspase-3

Lysates of cultured cells were prepared by washing cells twice in PBS, lysing cells in lysis buffer (10 mM Tris HCl, pH 7.4, 150 mM NaCl, 2% sodium dodecyl sulfate, 4 M urea), boiling for 5 min, and passing through a 26-g needle five times. Lysates of corneal tissue were prepared by excising whole cornea from a euthanized animal, mincing the tissue with a razor blade, and sonicating the tissue for 10 min. Sonicates were boiled in lysis buffer for 5 min and passed through a 26-g needle five times. Total cell protein was determined using the bicinchoninic acid (BCA) assay (Pierce Chemical). Samples were loaded onto 4% to 12% gradient bis-tris gels (Invitrogen) and proteins were separated by electrophoresis. Proteins were transferred to polyvinyl difluoride (PVDF) membranes (Invitrogen) by electrophoresis in NuPAGE transfer buffer (Invitrogen) with 10% methanol. Membranes were blocked in blocking buffer of 5% nonfat dry milk in TBS-T (10 mM Tris HCl, pH 7.4, 150 mM NaCl, 0.1% Tween-20), then incubated in polyclonal anti-active caspase-3 (BD Pharmingen) in blocking buffer for 30 min at room temperature. After washing in TBS-T, membranes were incubated in horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (BD Pharmingen) in blocking buffer. HRP labeling was detected using enhanced chemiluminescence (ECL; Amersham Pharmacia,
RESULTS

Apoptosis in Rejected Corneal Allografts
The occurrence of apoptosis in rejected C57BL/6 corneal allografts in BALB/c hosts was detected by in situ TUNEL assay. Syngeneic corneal grafts between BALB/c donor-host pairs (which never opacify and by definition do not reject) displayed no TUNEL staining in situ at any time point studied (Fig. 1A). LR corneal allografts, in which the host bed was not inflamed at the time of transplantation, displayed consistent but little TUNEL staining even when these grafts developed signs of rejection (Fig. 1B). In contrast, all HR corneal allografts, in which the host bed was inflamed and vascularized at the time of transplantation and which uniformly reject by day 18 to 20, displayed extensive TUNEL staining as early 7 days, with even more pronounced staining by day 14 after transplantation (Fig. 1c).

Lack of Apoptosis in Inflamed Corneas Not Receiving Allografts
Because HR corneal allograft beds are characterized by intense stromal infiltration and we consistently observed high levels of apoptosis only in HR grafts (of which nearly all reject), we examined whether the difference in apoptosis observed in the two (LR and HR) allograft groups was because of the level of corneal inflammation at the time of transplantation per se or the alloimmune response. Even after induction of intense inflammation in the stroma, but without transplantation, sutured inflamed corneas displayed virtually no TUNEL staining (as opposed to the HR graft) despite extensive corneal inflammation and neovascularization as confirmed by hematoxylin-eosin staining (Fig. 2), which suggests that corneal inflammation alone did not lead to appreciable keratocyte apoptosis.

Expression of Active Caspase-3 in Rejected Corneal Allografts
To confirm the above in situ results, rejecting (i) HR allografts, (ii) LR allografts, and (iii) ungrafted but inflamed BALB/c corneas, were subjected to SDS-PAGE and Western blotting for active caspase-3 expression. A polyclonal antibody that identifies both the 11 kDa and 17 kDa subunits of mouse-activated caspase-3 and the 18 kDa subunit of human active caspase-3 was used. Jurkat cells that had been treated with staurosporine for 4 hr were used as positive control for active caspase-3. The results confirmed the in situ TUNEL assay findings that rejecting C57BL/6 corneal allografts in HR BALB/c hosts contain apoptotic cells, as demonstrated by the presence of active caspase-3 in the corneal tissue (Fig. 3). In contrast, corneas taken from rejecting C57BL/6 corneal allografts in LR BALB/c hosts, or from highly inflamed but un-grafted BALB/c corneas, did not display active caspase-3 expression on Western blotting (Fig. 3).

Localization of Apoptosis to Resident Corneal Cells
The above results, showing that corneal allograft rejection in the HR setting triggers cellular apoptosis in the corneal stroma, led to the question of which cells were undergoing apoptosis, given that it has been demonstrated previously that invading leukocytes may undergo apoptosis in the anterior segment of the eye (7). However, our results suggest a lack of perfect convergence between (i) the area of maximal infiltration (mid and deep stroma) and apoptosis (anterior stroma), and (ii) between stomal infiltration per se and cell death (Figs. 2 and 3). Hence, we hypothesized that resident non-bone marrow-derived cells of the cornea, rather than the infiltrating leukocytes, were undergoing apoptosis. To test this hypothesis, rejecting C57BL/6 corneal allografts in HR BALB/c hosts were simultaneously examined for TUNEL staining and expression of CD45, a pan-leukocyte marker. The results demonstrated that
TUNEL staining and CD45 expression were minimally co-localized to the same cells in these HR recipients (Fig. 4a). The little TUNEL staining that was present in LR corneal allografts (Fig. 1) was not associated with cells expressing CD45 (data not shown). As a control, inflamed ungrafted BALB/c corneas were also double-stained with TUNEL and anti-CD45. No apoptotic cells were identified in inflamed ungrafted corneas, despite numerous CD45+ cells infiltrating the corneal stroma (Fig. 4b).

**Induction of Apoptosis in Keratocytes In Vitro**

Previous work in our laboratory had confirmed that one of the factors distinguishing the microenvironment of the HR allograft from that of the LR allograft is the profound overexpression of proinflammatory cytokines in the former compared with the latter (4,5). Given our results, and to elucidate potential apoptotic signals that could trigger apoptosis of corneal stromal cells seen in the HR allografts, cultured immortalized (donor-type) C57BL/6 keratocytes (MK/T-1 cells) were stimulated in vitro with various pro-apoptotic cytokines, alone or in combination. Expression of active caspase-3 was determined after 24 hr by Western blot analysis. The results indicate that ample Fas activation alone is not sufficient to stimulate caspase-3 activation, and thus apoptosis, in keratocytes (Fig. 5). A second or amplifying signal, in this case provided by pro-inflammatory cytokines interleukin-1β or tumor necrosis factor-α, was required concurrently with Fas activation to stimulate caspase-3 activation (Fig. 5).

**DISCUSSION**

Despite much progress in this area, the precise mechanisms of corneal allograft failure are not clear at this point; specifically, there is very little information as to how alloreactive cells effect graft decompensation and ultimate failure (6). In the present study, we used a well-established mouse model of orthotopic allogeneic corneal transplantation to study the role of apoptosis in corneal allograft failure, characterized by the loss of clarity in the stroma of the grafted tissue. We used two types of BALB/c corneal allograft recipients: LR recipients with no prior inflammation in the host bed that normally reject approximately 50% of grafts, and HR recipients with inflamed host beds in which nearly all grafts are rejected swiftly. Stromal cells from both HR and LR rejecting allografts were found to contain apoptotic cells by both in situ TUNEL staining (Fig. 1) and Western blot analysis of active caspase-3 in tissue lysates (Fig. 3); however, HR allografts had significantly higher numbers of apoptotic cells compared with their LR counterparts. Interestingly, however, our data clearly suggest that corneal inflammation alone, even accompanied by profound infiltration and neovascularization of the stroma, does not itself promote corneal cell apoptosis (Fig. 2). Therefore, the initiating apoptotic signal(s) may derive not just from the inflammatory response, but rather from the targeted and donor-specific alloimmune response generated against the transplant. This does not mean, however, that inflammation is not critical, as reflected by the profoundly increased level of apoptosis in the HR setting and the promotion of apoptosis by pro-inflammatory cytokines. Our data also strongly suggest that the grafting process alone does not promote apoptosis of corneal cells, as demonstrated by a lack of apoptotic cells in BALB/c to BALB/c syngeneic grafts (Fig. 1). We know this for several reasons. First, although the recipients of these syngeneic grafts had LR graft beds, previous work by our laboratory has shown that syngeneic corneal grafting uniformly produces far less pro-inflammatory cytokine response than does allogeneic grafting (5). Second, intense (but non-immunogenic) inflammation also fails to promote corneal apoptosis as shown in Figure 2, underscoring the relevance of allogeneic rather than non–antigen-specific inflammation in driving apoptosis.

Apoptosis has been shown to be a major factor in the maintenance of ocular immune privilege via the interactions of FasL on corneal endothelial cells and Fas on infiltrating, presumably alloreactive, T cells, resulting in T-cell apoptosis in the anterior chamber of the eye (7,8). T cells are also known to express FasL, and corneal endothelial cells and keratocytes are known
to express Fas (9-11); hence, a variety of cells (both mesenchymal and infiltrating bone marrow-derived) are capable of undergoing apoptosis via the Fas-FasL pathway. Our data clearly demonstrate that the potent allo-reactivity generated to HR grafts is a factor in inducing apoptosis. However, the fact that most stromal apoptotic cells are CD45− suggests that the FasL+ CD45+ infiltrating cells may promote donor tissue cell death by interacting with Fas+ keratocytes and by secreting cytokines (e.g., IFN-γ) that trigger or amplify apoptosis—similar to our in vitro immunoblot data (Fig. 5). In HR host beds, neovascularization of the cornea allows for an easy entry route for alloreactive T cells into the cornea to effect keratocyte apoptosis, which is normally not present at appreciable levels in LR host beds, thus explaining why HR grafts have significantly higher incidences of TUNEL+ cells and active caspase-3 expression compared with LR grafts. Recent data from our laboratory have also demonstrated distinctions in the pathways of allosensitization between HR and LR grafts, with direct alloreactive cells being primed only in HR recipients (12). The differential role, if any, of directly versus indirectly primed T cells in effecting apoptosis remains incompletely understood. By design, however, we focused this study exclusively on the role of apoptosis in the stroma of (target) corneal grafts, rather than the role of apoptosis in regulating the function or survival of FasL+ effector T cells. Hence, these data should be seen as neither refuting nor confirming the role of alloreactive T-cell apoptosis in regulating alloreactivity because most of these cells flow in the anterior chamber rather than infiltrate the stroma (7). The current results suggest a “bystander effect” of T cell-mediated apoptotic killing of corneal graft cells, in many ways reminiscent of the epidermal apoptosis seen in contact hypersensitivity (13). In the model we propose, FasL+ CD45+ cells infiltrating a HR allograft find themselves in a highly pro-inflammatory milieu, allowing a combined stimulus of pro-inflammatory cytokines and FasL to induce apoptosis of Fas+ corneal keratocytes. Our observation that Fas ligation alone did not induce apoptosis of keratocytes in vitro, but that it did in combination with IL-1β or TNF-α stimulation, supports this hypothesis.

We believe these data make a significant contribution to the complex question of corneal allograft rejection and represent a possible explanation for the increased risk and significant clinical burden of corneal allograft rejection in patients with HR inflamed host beds. Other studies have shown that corneal transplantation using wild-type donors and gld FasL-deficient hosts results in a significantly reduced risk of allograft rejection (14), suggesting that host FasL-donor Fas interactions are correlated with allorejection. However, in the same study, no difference in corneal allograft rejection was demonstrated between wild-type donor-host combinations and those involving lpr donors deficient in Fas and wild-type hosts, suggesting either that Fas-mediated cell death alone cannot fully account for graft failure or that compensatory mechanisms for apoptosis may be involved in the gld and lpr mouse strains. Additionally, the foregoing studies were performed in LR host beds and therefore the role of donor tissue Fas deficiency in modulating HR graft survival has yet to be determined. Studies from our laboratory have also shown that inhibiting pro-inflammatory cytokines in vivo, including IL-1 and TNF-α, have a significant effect in promoting corneal allograft survival (15-17). A possible, though as yet unconfirmed, mechanism suggested by our data is that anti-inflammatory cytokine strategies may promote graft survival, at least in part, by inhibiting the additional inflammatory signals required to induce corneal cell apoptosis.

Corneal transplantation remains the oldest successful, and most common, form of tissue grafting, and has restored sight to millions of blind individuals. Although a significant number of grafts are lost to rejection and chronic decompensation, the precise mechanisms behind graft failure remain elusive. Additional studies are required to further evaluate the role of inhibiting apoptosis at the level of the grafted tissue in suppressing the incidence of corneal graft failure.

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REFERENCES


FIGURE 1.
Apoptosis in syngeneic and C57BL/6 corneal allografts in low-risk and high-risk BALB/c hosts (representative micrographs shown). (A) In situ fluorescein TUNEL staining in syngeneic BALB/c to BALB/c corneal graft revealing no appreciable apoptosis. (B) Left, representative in situ fluorescein TUNEL staining in rejecting low-risk corneal allograft. Right, DAPI counterstain of same eye section. C) Left, in situ fluorescein TUNEL staining in high-risk corneal allograft on day 14 at the outset of the first signs of rejection, showing extensive apoptosis particularly in the anterior stroma. Right, DAPI counterstain of same eye section. Ep, epithelial layer; St, stromal layer. All micrographs taken at magnification ×400.
FIGURE 2.
Non-specific corneal inflammation alone does not induce stromal apoptosis. Left, in situ fluorescein TUNEL assay staining of BALB/c cornea on day 14 after placement of three intrastromal sutures to induce intense inflammation. Center, DAPI counterstaining of same eye section. Right, Hematoxylin-eosin stain of inflamed BALB/c cornea showing intense cellular infiltration and neovascularization. Arrows depict blood vessels with intense mononuclear cell infiltrate. Ep, epithelial layer; St, stromal layer. All micrographs taken at magnification ×400.
FIGURE 3.
Active caspase-3 expression in rejecting corneal allografts and inflamed BALB/c corneas. Corneas were removed after enucleation and cellular lysates were prepared for Western blotting. Mouse active caspase-3 polyclonal antibody staining was detected by HRP-conjugated anti-rabbit IgG and ECL detection. Jurkat cells served as controls. Results show expression of active caspase-3 in high-risk corneal allografts but not other experimental groups.
FIGURE 4.
Localization of apoptosis to non-bone marrow-derived cells. (A) Concurrent staining of high-risk corneal allograft with fluorescein TUNEL (green) and rhodamine anti-CD45 (red) reveals few double-labeled cells. (B) Double staining of inflamed but ungrafted BALB/c cornea reveals numerous CD45+ leukocytes but no TUNEL-labeled cells. Ep, epithelial layer; St, stromal layer. All micrographs were taken at ×400 magnification.
FIGURE 5.
Western blot analysis of active caspase-3 expression in stromal MK/T-1 cells after Fas and cytokine stimulation. Lanes: 1, Jurkat cells stimulated with staurosporine for 4 hr. 2-10, MK/T-1 cells stimulated for 24 hr with (2) media alone, (3) rat IgG, (4) 10 ng/mL Jo2, (5) 10 ng/mL IFN-γ, (6) 10 ng/mL IL-1β, (7) 10 ng/mL TNF-α, (8) Jo2 + IFN-γ, (9) Jo2 + IL-1β, and (10) Jo2 + TNF-α. Active subunits of mouse caspase-3 (p17 and p12) are identified by arrows.