Early ocular chemokine gene expression and leukocyte infiltration after high-risk corneal transplantation

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Purpose: To determine the differential gene expression of chemokine species and leukocyte infiltration of grafts in the early pre-rejection postoperative period after high-risk (HR) compared to normal-risk (NR) corneal transplantation.

Methods: Fully mismatched and syngeneic corneal grafts were performed in NR (avascular) and HR (vascularized) recipient beds of BALB/c murine hosts. Gene expression levels of a panel of chemokines were determined by a multiprobe ribonuclease protection assay system. The profiles of infiltrating cells into the corneal grafts at the same times were determined immunohistochemically.

Results: Compared to NR transplantation, HR eyes exhibited significantly higher mRNA levels for macrophage inflammatory protein (MIP)-2 and monocyte chemotactic protein-1 (MCP-1) on day 1, and for eotaxin on days 1 and 3 after transplantation. By day 6 after transplantation, still well before graft rejection, significantly higher levels of RANTES, eotaxin, MIP-1α, MIP-1β, MIP-2, and MCP-1 were detected in HR eyes. The overexpression of MIPs in HR eyes correlated with a significant increase in the number of infiltrating macrophages (p<0.01), and neutrophils (p<0.05) in HR compared to NR recipients. Low levels of eosinophil and mast cell infiltration were observed in all grafts, with a modest increase in mast cell infiltration (p<0.05) in HR compared to NR grafts.

Conclusions: These results suggest that increased expression of gene products for select chemokines, in particular those that mediate recruitment of innate immune cells, in the early period after HR corneal transplantation is related to the enhanced leukocytic infiltration of grafts observed in HR keratoplasty.

Chemokines represent a large superfamily (nearly 50 have been identified to date) of small molecular weight cytokines with highly conserved cysteine residues that are potent attractors of various leukocyte subsets including neutrophils, monocytes/macrophages, mast cells, lymphocytes, eosinophils, and dendritic antigen-presenting cells (APCs). Along with adhesion molecules, chemokines provide directional signaling for leukocyte trafficking and recruitment in immune-inflammatory responses in a highly coordinated multistep process [1]. In addition to their role in regulating inflammatory responses, chemokines also play critical roles in homing and recirculation of both naive and primed lymphocytes and are hence thought to be highly relevant to generation of adaptive immunity to foreign (including transplantation) antigens [1,2].

The prolonged survival of corneal transplants, reflected by the fact that nearly half of experimental fully allogeneic grafts survive indefinitely even without immunosuppressive therapy, has been related to ocular immune privilege and the induction of unique regulatory pathways that modulate both the induction (sensitization) and effector (rejection) phases of alloimmunity [3]. This relative success in normal-risk (NR) corneal transplantation, in which the donor tissue is grafted onto uninflamed avascular host tissue, is overshadowed by the very poor outcomes observed in high-risk (HR) corneal transplantation performed in inflamed and vascularized beds where the majority of grafts are lost to immune rejection in eyes that fail to exhibit important facets of immune privilege [4,5].

We have hypothesized that chemokines, as important mediators of the immune response, play a critical role in corneal alloimmunity. In a previous study from our laboratory in which chemokine expression was studied in rejected grafts and compared to nonrejected grafts at a later time, we reported that upregulation in gene expression of select CC (also known as β) and CXC (α) chemokines is closely associated with the effector phase of corneal transplant rejection in hosts receiving grafts in uninflamed NR beds [2]. However, earlier studies left open the question of which chemokines play crucial roles in immune and inflammatory cell recruitment in the early phase of the alloimmune response prior to development of graft rejection. Our previous work has shown that recipients of HR grafts develop allospecific sensitization at least 2-3 full weeks prior to hosts receiving NR grafts [6]. Specifically, HR murine hosts of corneal grafts exhibit delayed-type hypersensitivity (DTH)-type alloreactivity as early as 1-2 weeks post-keratoplasty compared to NR hosts that exhibit allospecific responses nearly 4 weeks after transplantation. The fact that corneal allosensitization can be universally demonstrated early and prior to development of graft rejection [7], allows for determining critical early-phase ocular mediators of alloimmunity prior to the possible confounding role of the rejection process itself on important facets of inflammation in the corneal tar-
get tissue. Accordingly, in the current series of experiments our main objective was to determine which ocularly expressed chemokines are particularly associated with the early recruitment of leukocytes in high-risk corneal grafts prior to development of graft rejection. Our results indicate that overexpression of macrophage inflammatory protein (MIP)-2, MIP-1α, MIP-1β, monocyte chemotactic protein (MCP)-1, eotaxin and regulated upon activation, normal T cell expressed and secreted (RANTES) genes is associated with the enhanced recruitment of leukocytes, in particular macrophages and to a lesser extent neutrophils and mast cells, to HR corneal grafts well prior to development of transplant rejection.

**METHODS**

**Animals:** BALB/c (H-2b) mice (Taconic, Germantown, NY) were used as recipients, and C57BL/6 (H-2b) mice (MHC and multiple minor H disparate) or BALB/c (syngeneic) corneas were used as donors. All experiments utilized male mice that were 8-12 weeks of age. All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Induction of high-risk corneal graft beds and orthotopic corneal transplantation:** Corneal neovascularization was induced by placement of intrastromal sutures to create high risk eyes as has been described previously [6] except for minor modifications. Briefly, 3-4 interrupted 11-0 nylon sutures were placed in the paracentral areas of the host cornea for 2 weeks prior to transplantation. Orthotopic penetrating keratoplasty was performed 5 days after the suture removal as described previously [2]. Briefly, the recipient cornea was marked with a trephine and excised with microscissors to a size of 1.5 mm. The donor cornea was excised with a 2.0 mm trephine (Storz Instrument Co., St. Louis, MO) and transplanted into the host corneal bed with 8 interrupted 11-0 nylon sutures (Sharpoint, Vanguard, TX). Eyes complicated with anterior synechiae were excluded from the study. No eyes exhibited any sign of infection. Eyes were enucleated and subjected to chemokine mRNA analysis and immunohistochemical study.

**RNA preparation and ribonuclease protection assay:** Total RNA was extracted by the single-step method using RNAzol B (Tel-Test, Inc., Friendswood, TX). Briefly, eyes were homogenized and centrifuged to remove cellular debris. The RNA pellet obtained from 4 eyes was resuspended in nuclease-free water and processed together as a group. Detection and quantification of murine chemokine mRNAs were carried out with a multiprobe ribonuclease protection assay (RPA) system (PharMingen, San Diego, CA) as recommended by the supplier. Briefly, a mixture of [α-32P] UTP-labeled antisense riboprobes was generated from the chemokine template set mCK-5 (PharMingen). Total RNA (20 µg) was used in each sample. Total RNA was hybridized overnight at 56 °C with 300 pg of the 32P-anti-sense riboprobe mixture. Nuclease-protected RNA fragments were purified by ethanol precipitation. After purification, the samples were resolved on 5% polyacrylamide sequencing gels. The gels were dried and subjected to autoradiography. Protected bands were observed after exposure of gels to x-ray film. Specific bands were identified on the basis of their individual migration patterns in comparison with undigested probes. The bands were quantitated by densitometric analysis (NIH Image, version 1.63) and were normalized to L32 (a ribosomal protein, a housekeeping gene) as previously reported [2]. The experiments were repeated twice. As the result of densitometric data, averaged density from two sets of RPA results was shown.

**Histochemical studies:** Mice were killed on day 1, 3, or 6 after allogeneic (5-6/time) or syngeneic (5-6/time) NR or HR transplantation and the enucleated eyes were used for histochemical studies. Cryosections (6 µm thick) were prepared extending transversely across the central cornea of each eye and fixed in acetone for 10 min. Sections were stained with respective antibodies to identify cell types: Fluorescein isothiocyanate (FITC)-conjugated anti-mouse F4/80 (BM8, 1:80; eBioscience, Inc., San Diego, CA) antibody to identify macrophages, FITC-conjugated anti-mouse CD3 (145-2C11, 1:100; BD PharMingen) antibody for T lymphocytes, and phycoerythin (PE)-conjugated anti-mouse Gr-1 (RB6-8C5, 1:200; BD PharMingen) antibody for neutrophils. The sections stained with FITC-conjugated antibodies were cover-slipped using an anti-fading mounting medium that contained propidium iodide (PI; Vectashield; Vector Laboratories, Peterborough, UK). Nuclear staining with PE-conjugated antibody was done by SYBR Green. Incubation with FITC anti-F4/80 antibody was performed overnight at 4 °C and all the other staining procedures were done at room temperature. Ten serial sections per specimen per stain were evaluated in a masked fashion, and the average number of F4/80-, Gr-1-, or CD3-positive cells per section under the 200x high power field averaged. The respective isotype controls for F4/80, CD3, and Gr-1 were FITC-conjugated rat IgG2a, hamster IgG, and rat IgG2b (all from Pharmingen). To evaluate the number of infiltrating eosinophils and mast cells, eyes were fixed in 5% formaldehyde for 24 h and sectioned. Staining was performed with Toluidine Blue and Congo Red, and the number of mast cells and eosinophils were similarly enumerated using a micron grid under 200x power field and averaged for ten serial sections per specimen.

In a separate series of studies, specimens harvested at day 6 were immunohistochemically studied to determine the protein expression of select chemokines in the cornea. Biotin-conjugated polyclonal antibodies for MIP-1α (goat IgG, 5 µg/ml; R&D Systems, Minneapolis, MN) and RANTES (goat IgG, 5 µg/ml; R&D Systems) were used for immunostaining of chemokine-expressing cells at day 6. An immunoperoxidase technique was performed as per the manufacturer’s instructions. Briefly, frozen specimens were sectioned into 6 µm specimens using a cryostat, fixed in acetone for 10 min, and washed with phosphate buffered saline (PBS). The antibodies were applied for 30 min, after which the antibody-labeled sections were exposed for 20 min to horseradish peroxidase-labeled streptavidin. The sections were incubated for 1 min in diamobenzidine and stained with Mayer’s hematoxylin for 10 s. Biotin-conjugated goat IgG (R&D Systems) was used as a negative control. Positive cells were counted per 200x high power field as described above.
Statistical analysis: The unpaired t-test (StatView version 5.0, Abacus Concepts, Inc., Cary, NC) was used to compare the number of infiltrating leukocytes and MIP-1α- or RANTES-positive cells in the corneas. Statistical tests were considered significant when p values were less than 0.05.

RESULTS
Chemokine gene expression in normal and high risk BALB/c hosts: We used whole eyes, not corneas, for isolation of total RNA, because more than ten corneas for a group are required for repeated RPA, and leukocyte infiltration into the posterior compartments of the eye was not observed after corneal transplantation (unpublished). Moreover, data on chemokine expression with whole eyes can reproduce the data with cornea only with the exception that eotaxin was not expressed in normal corneas [2]. Figure 1 shows the representative results of the chemokine RPA autoradiography, and Figure 2 the averaged quantity from two sets of RPA results of chemokine mRNA normalized to L32 at different times. NR corneal transplantation of either allogeneic or syngeneic tissue led to modest increase of RANTES, MIP-1α, MIP-1β, MIP-2, and MCP-1 mRNA expression in the early postoperative period. There was no appreciable difference in the levels of chemokine gene expression between syngeneic (isograft) and allogeneic grafting in this time period. Eotaxin mRNA was expressed constitutively in normal unoperated control eyes and HR eyes at day 0, and the expression levels gradually increased during the follow-up period (Figure 2).

Except for lymphotactin and TCA-3, which were undetectable in all of the samples evaluated (Figure 1), and IP-10, which was not detected until day 6 after surgery, there was overexpression of all other chemokines in particular eotaxin, MIP-2, and MCP-1 by 24 h after HR keratoplasty (Figure 2). Elevated levels of chemokine expression were detected in HR grafts on day 3 after surgery, although levels of MIP-2 and MCP-1 lowered modestly by day 3 but remained elevated for eotaxin. By day 6 after transplantation there was an increase of MIP-1α, MIP-1β, MIP-2, eotaxin, MCP-1, and RANTES mRNA expression in HR compared to the NR grafted eyes. Minimal expression of IP-10 mRNA was detected in the HR grafted eyes, and only on day 6. Finally, to determine to what extent the increased expression of various chemokine species was a function of the inflamed host bed, gene expression was compared between allogeneic and syngeneic transplantation in HR eyes (4 per group) at day 6. Similar to our findings in the NR setting, levels of chemokine gene expression at day 6 were indistinguishable between isografted and allografted eyes (data not shown).

Profile of corneal leukocyte infiltration: To delineate the differential expression of inflammatory chemokines in HR keratoplasty with alterations in leukocytic infiltration, immunohistochemical techniques were employed to enumerate the

![Figure 1. Chemokine gene expression during the early postoperative phase after corneal transplantation. Total RNA (20 µg) was applied to each lane to quantify the gene expression for nine chemokine species at each of 4 times (day 0 data represent nonsurgical eyes). Two sets of RPA results were obtained, and representative autoradiographic data for the panel of chemokines tested are shown. On the basis of the undigested probes’ migration patterns, specific bands for each chemokine are identified as shown for ungrafted normal corneas, isografts, normal-risk allografts, and high-risk allografts at the specified times after transplantation.](http://www.molvis.org/molvis/v11a75/634)
number of macrophages, T cells, and neutrophils based on membrane expression of F4/80, CD3, and Gr-1, respectively, on day 3 (Figure 3) and day 6 (Figure 4) after corneal transplantation. Additional histochemical analyses were performed to enumerate the number of mast cells and eosinophils infiltrating corneal grafts in the early postoperative period. The predominant infiltrating cell type, at both times, for all studied groups, was F4/80+ cells (macrophages; p<0.01), followed by CD3+ (T) cells, mast cells, Gr-1+ cells (neutrophils), and eosinophils. Statistical analyses of the data did not reveal any significant difference in the number of infiltrating cells in these early time periods among NR isografts compared to NR allografts. However, there was a significant increase in the number of infiltrating leukocytes, in particular macrophages (p<0.05, day 3; p<0.01, day 6) and to a lesser extent neutrophils (p<0.05) and mast cells (p<0.05; Figure 5), in HR grafts compared to the NR transplants. Representative photographs of neutrophils and macrophages were shown in NR and HR grafts (Figure 6). No significant difference of leukocyte infiltrating number between NR and HR allografts was observed on day 1 (data not shown).

Immunohistochemical study of chemokine protein expression: We have previously reported on the overexpression of ligands to CCR1 and CCR5 receptors in rejecting corneal allografts [2]. We therefore hypothesized that HR keratoplasty is associated with enhanced presence of cells expressing RANTES and MIP-1α, two principal ligands binding CCR1 and CCR5 on mononuclear cells. Interestingly, while our results for RANTES showed insignificant differences in the number of cells expressing this chemokine between HR and NR allografts in the early postoperative period (p>0.1), there was significantly enhanced expression of MIP-1α-expressing cells, primarily macrophages, in HR grafts (Figure 7). The number of MIP-1α positive cells per 200x high power field was 3.8±1.4 (mean±SD) in HR allografts compared to 0.8±0.6 in NR allografts (p<0.05), and 0.7±0.4 (p<0.05) in NR isografts.

**DISCUSSION**

HR corneal transplantation presents a significant challenge due to efficient induction of alloimmunity in the host [4-6], and rejection of the vast majority of grafts by alloreactive effector cells [8-10]. In the current study we evaluated differential gene expression of a panel of chemokines in the early postoperative period after high-risk keratoplasty, hypothesizing that an increase in expression of select chemokine species is associated with the early infiltration of high-risk grafts. Our data in fact suggest that as early as 24 h after HR, but not NR trans-
plantation there is enhanced expression of MCP-1, eotaxin, and MIP-2 (mouse homolog of IL-8) mRNA. This is followed by a significant increase in expression of a wider spectrum of chemokine genes including RANTES, MIP-1α, and MIP-1β, which correlate with increased leukocytic infiltration of HR grafts. Our current observations are in accord with other data from our [6,11] and other laboratories [5,12] which in the aggregate suggest that enhanced expression of proinflammatory cytokines such as interleukin (IL)-1 and tumor necrosis factor (TNF)-α in the inflamed corneal bed provides, at least in part, the molecular basis for the tissue infiltration observed in high-risk transplantation [11,13]. Activation of the NF-κB signal transduction pathway, which is associated with ligation of IL-1 and TNF-α receptors, is one of the most critical events that triggers cellular responses in inflammation, including enhanced transcription of select “inducible” proinflammatory chemokine genes such as MIP-2, MIP-1α, and RANTES [1,14,15]. We propose, therefore, that our current data represent the chemokine counterpart to the well-described inflammatory cytokine increase in expression in the microenvironment of HR corneal grafts.

The chemokine data presented here provide important clues as to the key mediators of alloimmunity in high-risk keratoplasty. A large pool of data suggest that the immune mechanism that destroys corneal allografts is largely mediated by host T helper-1 (Th1) cells that effect DTH against donor cells [2-4,6,7,9], and the early rejection of high-risk grafts has been related to the early induction of allospecific DTH [6,10]. Chemokines that mediate DTH reactivity include MIP-1α, MIP-1β, and RANTES that bind CCR1 and CCR5 receptors [1,15]. Consistent with the early induction of allospecific DTH in hosts of HR grafts, our data suggest significantly elevated levels of these Th1 type chemokine mRNA species in the early postoperative period after HR transplantation. It is noteworthy, however, that the two chemokine species that are particularly upregulated by 24 h after HR transplantation are MCP-1 and MIP-2, critical chemoattractants for innate immune cells such as macrophages and neutrophils, respectively [1,2]. Since there is only limited leukocytic infiltration of grafts immediately after surgery [11,13], it is likely that the very early (the first 24 h) chemokine expression after HR transplantation is generated by resident corneal cells that are nevertheless capable of attracting innate proinflammatory...
cells (macrophages and neutrophils). This hypothesis is supported by data demonstrating the capacity of corneal cells themselves to secrete chemokines [16], and indirectly supported by the bimodal infiltration of the cornea by innate inflammatory cells [11] which corresponds with the bimodal (day 1 and day 6) enhanced secretion of chemokine species such as MIP-2 and MCP-1 that mediate neutrophil and macrophage recruitment. The early influx of innate immune cells in response to ocularly-generated chemokines can in turn lead to a second wave of inflammatory chemokine expression (e.g., MIP-1α, RANTES) and resultant cell recruitment by day 6, reminiscent of the multiple waves of cornea-infiltrating cells mediated by different chemokines in herpetic keratitis [16]. In comparison with the high number of macrophages detected in corneal graft, infiltrating neutrophils are few in the postoperative early phase of corneal transplantation. This may be because a significant number of macrophages are constitutively distributed in normal mouse cornea as previously described [17-19].

Figure 5. Enhanced presence of mast cells in high-risk transplantation. Representative photomicrograph of corneas harvested from normal-risk (NR; A) and high-risk (HR; B) transplant recipients 6 days post-keratoplasty. Toluidine blue staining of the sections reveals mast cells with characteristic magenta staining metachromatic granules (arrows). Increased infiltration of mast cells is observed into HR grafts (B) compared to NR grafts (A). Ungrafted normal corneas had no mast cells (not shown).

Figure 6. Neutrophil and macrophage infiltration into corneal allografts on day 6. Representative photomicrograph of corneas harvested from normal risk (NR) and high risk (HR) transplant recipients 6 days post-keratoplasty. Representative photographs using anti-Gr-1, anti-F4/80, and nonimmunized control rat IgG2a antibodies were shown. Increased infiltration of neutrophils (Gr-1; red) is observed into the HR grafts compared to the NR grafts. The number of macrophages (F4/80; green) in the HR grafts is high compared with the NR grafts. No positive staining in the NR grafts and the HR grafts were observed with FITC-conjugated rat IgG2a control antibody. SYBR Green (green; top two panels) and PI (red; lower 4 panels) were used for nuclear staining. No positive staining was observed with FITC-conjugated hamster and rat IgG2b control antibody (not shown).
In addition to demonstrating differential and time-dependent chemokine expression after HR keratoplasty, our data provide some clues as to the interplay between innate and adaptive immunity generated after corneal transplantation. Specifically, it is noteworthy that we find macrophages to be the dominant leukocyte subset in infiltrating HR grafts in the early postoperative phase. It is relevant that hosts of HR grafts do not demonstrate any DTH alloreactivity until the second week after transplantation [6], suggesting that macrophages may play an important role in early sensitization of graft recipients to donor transplantation antigens. These observations are compatible with the findings of Van der Veen et al. [19] and Torres et al. [20] that suggest a significant role for macrophages in corneal alloimmunity. Torres and coworkers showed a suppressed Th1 response in hosts treated with clodronate-containing liposomes [20], similarly suggesting a role for macrophages in induction of DTH-type alloimmunity. Given that macrophages express class II MHC and can serve as APCs [20,21], it is conceivable that their enhanced role in high-risk keratoplasty is critical in the efficient induction of allosensitization so characteristic in HR transplantation [6]. Such a model essentially proposes a “cross-talk” between innate and adaptive immune mediators wherein the early recruitment of innate immune cells into the corneal graft contributes to the adaptive T cell-mediated allospecific response that follows due to secretion by inflammatory cells of a wide array of chemokines (e.g., MIP-1α) whose main function is to chemoattract CD3 positive T cells. This proposed paradigm is supported by our observation of significant numbers of MIP-1α-secreting monocytes-macrophages in the early days after HR corneal transplantation.

Our previous work did not suggest a significant role for eotaxin in NR transplant immunity several weeks after grafting [2]. However, the data presented herein show a consistently elevated level of expression of eotaxin after HR transplantation. Corneal stromal cells can express high amounts of eotaxin with cytokine stimulation [22,23]. Eotaxin preferentially binds CCR3, which is expressed at very high levels on eosinophils and mast cells, and as such is an important mediator of Th2 inflammatory responses [1,15]. The associated increased infiltration of HR grafts by mast cells could be an “epiphenomenon” that may not be significantly relevant to alloimmunity. Alternatively, it may suggest that non-Th1-mediated mechanisms may be relevant in corneal alloimmunity as has been suggested recently by Niederkorn et al. [24] and has definitely been established for heart grafts [25]. Mast cells have not been systematically studied in corneal alloimmunity but are nearly ubiquitous in a wide variety of inflammatory responses as cells that facilitate the link between innate and adaptive immunity [26]. Recently, histamine, a major product of mast cells has been reported to induce CD86 (B7.2) costimulatory molecule expression by dendritic cells [27], the professional APCs of the cornea and ocular surface [28]. While we do not have any data to definitively demonstrate the interplay between mast cell activity and APC function in the cornea, it is possible that the enhanced presence of mast cells in HR grafts is associated with a more efficient maturation and sensitizing capacity of ocular APC by upregulating their expression of B7 costimulatory molecules.

Our previously published work on normal-risk grafts at later times conclusively showed that there are significant differences in ocular chemokine expression between isografts and allografts [2]. In contrast, our current data from the first week after keratoplasty suggest that the main determinant of

Figure 7. Enhanced presence of MIP-1α expressing cells in high-risk transplantation. These are representative micrographs of MIP-1α positive cells from high-risk grafts (HR; A), normal-risk grafts (NR; B), and HR grafts with isotype control antibody (C). There were significantly more MIP-1α positive infiltrating cells in the HR grafts than in the NR grafts.
chemokine expression is the degree of inflammation in the recipient bed (i.e., whether the host is normal or high risk) and not on the presence of allogeneic disparity per se. This suggests that the early overexpression of inflammatory chemokines after corneal transplantation is less a function of alloantigen-induced stimulation than of inflammation in the graft bed itself. The fact that the degree of inflammation in the host bed prior to transplantation can so profoundly affect the profile of chemokines expressed in the grafted eye is further testimony to the distinct innate molecular mechanisms that dictate inflammation in the early period after transplantation compared to those adaptive mechanisms that mediate T cell allospecific effector functions including rejection at later times.

Current immunosuppressive and immunomodulatory pharmaceuticals used in the prevention and treatment of corneal allograft rejection have significant limitations from the standpoint of both efficacy and side effects. This is felt nowhere more acutely than in high-risk transplantation where corneal allografts placed in inflamed and neovascularized beds are often rejected swiftly even with intensive corticosteroid therapy [4-6]. This represents the first study, to our knowledge, evaluating the differential expression of chemokines after HR transplantation. Still, we cannot as of yet propose specifically how the alloimmune response to high-risk corneal grafts can be altered by strategies that target one or another chemokine. We anticipate, however, that these data will take us yet one step closer to the eventual development of molecular or gene-based interventional strategies that can effectively prolong graft survival without the deleterious side effects of currently available nonspecific immune suppression.

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