Topical Soluble Tumor Necrosis Factor Receptor Type I Suppresses Ocular Chemokine Gene Expression and Rejection of Allogeneic Corneal Transplants

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**Objective:** To determine the effect of topical soluble tumor necrosis factor receptor type I (sTNFR-I) on survival of murine orthotopic corneal transplants and on ocular chemokine gene expression after corneal transplantation.

**Methods:** BALB/c mice (N = 50) were used as recipients of multiple minor H–disparate corneal transplants from B10.D2 donors. After orthotopic corneal transplantation, mice were randomized in a masked fashion to receive either topical sTNFR-I or vehicle 3 times daily, and all grafts were evaluated for signs of rejection and neovascularization by slitlamp biomicroscopy for 8 weeks. Ocular chemokine gene expression in sTNFR-I– and vehicle-only–treated groups was determined using a multiprobe ribonuclease protection assay.

**Results:** Hosts treated with topical sTNFR-I experienced significantly enhanced corneal allograft survival compared with animals treated with vehicle alone (P = .01). Moreover, postoperative messenger RNA levels of RANTES and macrophage inflammatory protein-1β in sTNFR-I–treated eyes were substantially suppressed compared with vehicle-treated eyes. Vehicle-treated eyes bearing rejected allografts expressed higher levels of messenger RNA for both chemokines than control eyes bearing accepted allografts.

**Conclusions:** Topical treatment with sTNFR-I promotes the acceptance of allogeneic corneal transplants and inhibits gene expression of 2 chemokines (RANTES and macrophage inflammatory protein-1β) associated with corneal graft rejection.

**Clinical Relevance:** Our findings support the feasibility of a topical anticytokine strategy as a means of reducing corneal allograft rejection without resorting to the use of potentially toxic immunosuppressive drugs.


Despite the overall success of corneal transplantation, immunologic rejection remains the principal threat to allograft longevity. Although not all of the cellular and molecular components that mediate corneal graft rejection have been identified to date, there is increasing evidence that the proinflammatory cytokine tumor necrosis factor α (TNF-α) is involved in the alloimmune response. Tumor necrosis factor α is a macrophage-derived cytokine that mediates many proinflammatory and immune regulatory functions, such as up-regulation in expression of adhesion and costimulatory molecules, neutrophil activation, induction of chemokine secretion, and activation of the nuclear factor–κB signal transduction pathway. Tumor necrosis factor α activity is regulated by 2 distinct receptors—type 1 (p55) and type II (p75)—which have largely homologous extracellular domains but distinct intracellular domains that can mediate discrete cellular responses. Tumor necrosis factor receptor type 1 (TNFR-I) is believed to be the principal receptor through which many of the proinflammatory activities of TNF-α are mediated.

In the setting of allogeneic corneal transplantation, there is profound and sustained up-regulation in expression of TNF-α protein and messenger RNA (mRNA) levels. The functional relevance of TNF-α in corneal transplant immunology has been shown recently by Yamada et al, who have shown that animals with gene-targeted deficiency in TNF-α accept minor H–disparate grafts at a significantly higher rate than wild-type controls. Moreover, animals with gene-targeted deficiency in TNF-α have suppressed capacity for corneal and limbal recruitment of Langerhans cells, suggesting that ligation of TNF-α can play a critical role in mediating important corneal immune functions.

Based on these data, it has been proposed that TNF-α could serve as an appropriate target for therapeutic intervention in prevention of corneal allograft
MATERIALS AND METHODS

ANIMALS

Six- to 8-week-old male BALB/c mice (Taconic Farms Inc, Germantown, NY) and B10.D2 mice (The Jackson Laboratory, Bar Harbor, Me) were used as graft recipients and donors, respectively. All animals were treated according to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

PHARMACOLOGICAL STRATEGY

One drop (5 µL) of each topical preparation was applied to BALB/c recipient eyes 3 times a day for 8 weeks starting 24 hours after transplantation. The experimental therapy comprised polyethylene glycolylated truncated monomeric recombinant methionyl human sTNFR-I (15 mg/mL) (Amgen Inc, Thousand Oaks, Calif) in 0.1% sodium hyaluronate in phosphate-buffered saline solution. Vehicle-treated animals received 0.1% sodium hyaluronate only.

CORNEAL TRANSPLANTATION

As described previously, each recipient was anesthetized with an intraperitoneal injection of 3 mg of ketamine hydrochloride and 0.0075 mg of xylazine hydrochloride before all surgical procedures. The central 2 mm of the donor cornea was excised and secured in recipient graft beds with 8 interrupted 11-0 nylon sutures (Sharpoint; Vanguard, Houston, Tex). Antibiotic ointment was applied to the corneal surface, and the lids were closed for 24 hours with an 8-0 nylon tarsorrhaphy. All grafted eyes were examined after 72 hours; no grafts were excluded from analysis because of technical difficulties. Transplant sutures were removed from all animals on day 7.

EVALUATION OF ORTHOTOPIC CORNEAL TRANSPLANTS

Grafts were evaluated for signs of rejection by slitlamp microscopy twice weekly for 8 weeks. At each evaluation the grafts were scored for opacity and neovascularization (NV). A previously defined and standardized scoring system was used to grade the degree of opacification from 0 to 5+ (0 indicates clear graft; 1+, minimal superficial opacity; 2+, mild stromal opacity with pupil margin and iris vessels visible; 3+, moderate stromal opacity with only pupillary margin visible; 4+, intense stromal opacity with the anterior chamber visible; and 5+, maximal corneal opacity with total obscuration of the anterior chamber). Grafts with an opacity score of 2+ or higher after 3 weeks were considered to be rejected; grafts with an opacity score of 3+ or higher at 2 weeks that never cleared were also regarded as rejected. Neovascularization was graded between 0 and 8 based on the degree of centripetal ingrowth and quadratic involvement of the neovessels.

RIBONUCLEASE PROTECTION ASSAY FOR CHEMOKINE GENE EXPRESSION

Our method of quantifying chemokine gene expression has been described elsewhere. Total RNA was extracted by the single-step method using RNAzol B (Tel-Test Inc, Friendswood, Tex). Eyes were enucleated 5 weeks after transplantation, homogenized, and centrifuged to remove cellular debris. The RNA pellet obtained from 3 eyes was resuspended in nuclease-free water and processed together as a group. Detection and quantification of murine chemokine mRNAs were accomplished using a multiprobe ribonuclease protection assay system (PharMingen). Fifteen micrograms of total RNA was used in each sample. Total RNA was hybridized overnight at 56°C with 300 pg of the 32P antisense riboprobes generated from the chemokine template set mCK-5 (PharMingen). Fifteen micrograms of total RNA was used in each sample. Total RNA was hybridized overnight at 56°C with 300 pg of the 32P antisense 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 compared with the undigested probes. The bands were quantitated by densitometric analysis (National Institutes of Health Image, Bethesda, Md) and normalized to glyceraldehyde-3-phosphate dehydrogenase. All samples were analyzed in triplet.

STATISTICAL ANALYSIS

Rates of corneal graft survival were plotted as Kaplan-Meier survival curves and were compared using the log-rank (Mantel-Cox) test. Comparison of corneal NV scores was conducted using the χ2 test. Chemokine mRNA expression values are given as mean ± SE. Comparison of chemokine expressions in vehicle- and sTNFR-I–treated groups was analyzed using 1-way analysis of variance and the Fisher exact test. Statistical significance was defined as P<.05.

rejection. It has already been shown that administration of anti–TNF-α therapy can be effective in treatment of immune-mediated diseases, including arthritis and some forms of uveitis, although inhibitors of TNF-α do not block endotoxin-induced uveitis. Therapy with anti–TNF-α antibody has also been shown to be effective in a rat model of cardiac allotransplantation. Because systemic administration of immune-modifying agents can be associated with untoward adverse effects, in this study we investigated the effect of topical soluble TNFR-I (sTNFR-I) on the survival of corneal allografts. Soluble TNFR-I has been shown to profoundly suppress bioactivity of TNF-α by binding free TNF-α and preventing ligation of the membrane-bound receptors.

Corneal allograft rejection is pathologically characterized by leukocytic infiltration into the graft stroma and adherence of mononuclear cells to the donor corneal endothelium. Recruitment of immune and inflammatory cells to the target tissue, including allografts, has been associated with the function of specialized chemotactic cytokines known as chemokines. Studies have shown that corneal transplant rejection is associated with profound up-regulation in ocular gene expression of CC (β) chemokines, including macrophage inflammatory protein-1 alpha microarray analysis (National Institutes of Health Image, Bethesda, Md) and normalized to glyceraldehyde-3-phosphate dehydrogenase. All samples were analyzed in triplet.

sTNFR-I could alter gene expression of the chemokines associated with corneal transplant rejection. Because postkeratoplasty NV might facilitate expression of immune and inflammatory responses at the graft site, we were interested in examining whether treatment with sTNFR-I imposed any appreciable effect on corneal NV scores after transplantation. During 8-week follow-up (Figure 2), corneas treated with sTNFR-I had generally a similar corneal NV score distribution pattern as that observed in corneal grafts treated with vehicle alone (P > .05). The exception is 3 weeks after transplantation, at which point sTNFR-I showed a marginal angiostatic effect such that 60% of vehicle-treated corneas had an NV score greater than 2 as opposed to only 27% of sTNFR-I–treated corneas (P = .047).

CHEMOKINE EXPRESSION

Figure 3 shows the mRNA levels of chemokines in the ribonuclease protection assay autoradiograph, and Figure 4 reflects the normalized densitometric quantification of RANTES and MIP1β gene expression levels. Control eyes revealed significant baseline expression of eotaxin and marginal expression levels of RANTES and lymphotactin mRNA. Compared with naive (ungrafted) control eyes, vehicle-treated eyes significantly overexpressed RANTES and MIP1β, with mRNA levels of both chemokines being modestly higher in eyes with rejected compared with accepted grafts (Figure 4). Comparison of sTNFR-I–treated grafted eyes with the 2 vehicle-treated groups revealed significant decreases in RANTES and MIP1β mRNA levels (P < .01 and P < .05, respectively). In fact, suppression of RANTES and MIP1β gene expression by sTNFR-I to levels below those seen for accepted vehicle-treated eyes suggests that depressed levels of mRNA for these chemokines are not entirely a result of graft acceptance and immunologic quiescence. The mRNA levels of RANTES and MIP1β in the sTNFR-I–treated grafted eyes were indistinguishable from those in naive controls. Comparison of other chemokine mRNA levels in vehicle-treated and sTNFR-I–treated grafted eyes did not reveal any statistically significant differences.

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a genetic deficiency of TNFR-I exhibit a profound increase in the survival rate of minor H–mismatched corneal grafts, strongly confirm TNF-α as an important mediator in the pathogenesis of corneal allograft rejection. We focused our attention on alloimmunity to minor antigens because it has been shown that disparity at the level of major histocompatibility complex in addition to minor antigens provides a significantly more formidable immune barrier to corneal graft acceptance than disparity at the level of minor antigens because it has been shown that disparity at the level of major histocompatibility complex in addition to minor antigens provides a significantly more formidable immune barrier to corneal graft acceptance than disparity at the level of minor antigens.

The precise action of TNF-α on the induction and expression of corneal alloimmune mechanisms remains incompletely understood. In the murine orthotropic corneal transplantation model, peak secretion of TNF-α protein in allogeneic grafts is observed 1 to 2 weeks after surgery, whereas graft rejection typically occurs at 4 to 5 weeks. Similarly, up-regulation of TNF-α mRNA in grafts, and increased levels of circulating TNF-α in host serum, is generally detected several weeks before graft rejection. In contrast, Larkin et al showed increased expression of TNF-α by alloreactive cells. Therefore, in the aggregate, these findings suggest that TNF-α might play an important role in the induction and expression phases of the alloimmune response. Involvement of TNF-α in the induction phase of alloimmunity is supported by previous data from our laboratory showing that the migration of professional antigen-presenting cells (including Langerhans cells) into the cornea is largely mediated by TNF-α. The critical role of antigen-presenting cell migration in indirect sensitization of T cells to corneal transplants is emphasized by data showing that suppression of Langerhans cell trafficking into corneal grafts can prevent host sensitization to the transplants. Accordingly, we propose that local neutralization of TNF-α activity by application of sTNFR-I imposes its beneficial effect on allograft survival, at least in part, by inhibiting leukocyte recruitment in the early postoperative period.

As shown previously, TNF-α need not effect its chemotactic activity on lymphoreticular cells directly. Tumor necrosis factor α is a pleiotropic cytokine whose activity is intimately related to a variety of nuclear factor–κB response elements, including chemokines and adhesion factors that can themselves mediate leukocyte recruitment. In fact, recent studies have indicated that expression of a variety of chemokines, including those operative on the ocular surface, can be under the regulation of locally produced TNF-α. To test whether topi-
cal application of sTNFR-I could down-modulate gene expression of inducible proinflammatory chemokines, we quantified chemokine mRNA in grafted hosts treated with sTNFR-I and vehicle alone. Consistent with previous findings in major histocompatibility complex and minor H fully mismatched corneal transplantation, increased gene expression of RANTES and MIP1β is associated with rejection of minor H–disparate corneal grafts. More important, our results clearly show that sTNFR-I therapy applied after corneal transplantation significantly down-regulates local gene expression of RANTES and MIP1β. These ligands, by virtue of binding to the widely expressed CCR1 and CCR5 receptors, serve as critical chemoattractants for antigen-presenting cells and activated CD4⁺ T cells. Preliminary data from our laboratory in nontransplant models of corneal inflammation show a significant suppression of corneal dendritic cell migration in response to corneal injury after treatment with sTNFR-I (unpublished observations). Our present data demonstrating that sTNFR-I treatment can suppress expression of RANTES and MIP1β below that for accepted untreated corneas suggest that the depressed level of chemokine expression is not simply secondary to suppression of the rejection process but rather a mechanism by which sTNFR-I can mediate its beneficial effect on allograft survival. Taken together, these data support our hypothesis that sTNFR-I can down-modulate the induction and expression phases of alloimmunity by suppressing leukocyte recruitment to the graft site.

It is important to underscore the limitations of this study. First, we do not have any data on intraocular penetration of sTNFR-I. The transcorneal penetration of high-molecular-weight proteins, including the nearly 50-kd polyethylene glycolylated monomeric TNF-I used in these experiments, is theoretically limited. However, several factors should be kept in mind. First, as previous work has suggested, high transcorneal penetration is not mandatory for down-modulating alloimmune responses. Second, the transcorneal penetration of molecules can be significantly altered (increased) through inflamed corneas because compared with normal corneas, their epithelial barrier does not maintain the same degree of impermeability. Finally, the fact that application of sTNFR-I led to modulation in alloimmunity and ocular chemokine gene expression offers strong support for its in vivo bioactivity.

The second limitation of our study is that we used whole-eye homogenates for analysis of chemokine mRNA to circumvent problems faced with the small quantities of RNA extractable from the murine cornea. Although this method does not allow for precise localization of mRNA expression (to the cornea), it has the benefit of allowing simultaneous quantification of different RNA species from the same ocular samples. Moreover, previous studies have shown an almost indistinguishable pattern of chemokine gene expression when RNA is extracted from whole eyes compared with exclusively from the cornea after transplantation. In addition, whereas leukocyte infiltration into the posterior compartments of the eye is not observed after corneal transplantation, effector cells involved in mediating graft rejection are commonly seen in noncorneal structures of the anterior segment, such as the anterior chamber and iris, most likely as a result of extravasation and recruitment at the level of the ciliary body and iris root. It is therefore likely that noncorneal structures of the anterior segment actively contribute to leukocyte recruitment by expressing chemokines. Therefore, although analysis of whole eyes has the disadvantage of not limiting the assay to the cornea alone, it has the advantage of assaying chemokines expressed by other structures in the anterior segment that probably play a functionally relevant role in leukocyte recruitment after corneal transplantation.

The third limitation of this study is that we restricted our evaluation of chemokine expression to mRNA, and not protein, levels. To the extent that the biological function of these factors depends on ligation of membrane-bound receptors, it is important to emphasize that differential levels of mRNA expression cannot be equated with protein bioactivity. However, ribonuclease protection assay and immunohistochemical data from our laboratory (unpublished observations) show a close temporal correlation between chemokine gene and protein expression in corneas receiving no immunomodulatory treatment. Therefore, it is likely that the suppressed gene expression of select chemokines by sTNFR-I also leads to down-modulation in their protein expression.

The role of TNF-α in corneal angiogenesis remains poorly understood. Inflammatory mediators have been implicated as critical factors for endothelial cell proliferation and angiogenesis. Our data do not show a significant effect of sTNFR-I treatment in postkeratoplasty NV. This is particularly striking given the positive effect of sTNFR-I administration on graft survival. However, it has been previously reported that postkeratoplasty NV and alloimmunity can significantly diverge. Hence, reduction in immune responses to the graft need not correlate with the degree of corneal NV. In any case, our data strongly suggest that, at least in the setting of transplantation, TNF-α antagonism with topical sTNFR-I therapy has no significant angiostatic effects.

To our knowledge, this represents the first study providing evidence for local anti-TNF strategies, using the novel method of topically administering sTNFR-I, for effective prevention of corneal allograft rejection. Various forms of recombinant sTNFR-I, including monomeric 4 domain, monomeric 2.6 domain, and 30-kd polyethylene glycol–linked 2.6 domain molecules, have been constructed. Although they differ in the number of domains and chemical modification, all forms of sTNFR-I have been shown to neutralize TNF-α activity and to efficiently inhibit experimental arthritis. We selected polyethylene glycol–linked sTNFR-I because this molecule has a long half-life and is intended for clinical use. Our choice of sodium hyaluronate as a vehicle was based on the fact that 0.2% sodium hyaluronate is a molecule with a long contact time on the ocular surface that is also well tolerated because of its biophysical properties and hence can increase the availability of pharmacotherapeutic agents to the ocular surface and cornea.

Currently available preventive and therapeutic regimens for corneal transplant rejection are associated with significant complications. Hence, it is desirable to devise intervention strategies that can prolong graft sur-
vival by specifically targeting molecules involved in generation of the alloimmune response. A variety of successful experimental strategies have been developed, including induction of tolerance to donor corneal cells<sup>44</sup>, macrophage depletion<sup>55</sup>, deviation of recipient immune systems toward Th2 response<sup>66</sup>, and intervention of the function of adhesion molecules, cytokines, or CD4<sup>+</sup> T cells. <sup>57-59</sup> Our data indicate that local neutralization of TNF-α activity also holds promise as an effective modality for suppressing TNF-α-mediated processes in the context of corneal transplantation. Further studies are required to better delineate the effect of topical sTNFR-I on cellular and molecular mechanisms of corneal immunity.

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