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 (MIP-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 MIP-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.

immune-mediated diseases, including arthritis, and anti–TNF-α antibody has also been shown to be associated with untoward adverse effects, in this study, systemic administration of immune-modifying agents can be effective in prevention and reversal of rejection episodes associated with the function of specialized chemotactic cells to the target tissue, including allografts. Therapy with anti–TNF-α antibody has also been shown to be effective in prevention and reversal of rejection episodes 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, macrophage inflammatory protein-1β...
(MIP1β), and RANTES,26 that are implicated in activation and selective recruitment of TH1 lymphocytes.27 Because TNFα, along with interleukin 1 and lipopolysaccharide, is one of the main stimuli for expression of a wide array of chemokines, including proinflammatory CC chemokines, we were also interested in determining whether local suppression of TNFα activity by application of sTNFR-I could alter gene expression of the chemokines associated with corneal transplant rejection.

RESULTS

ORTHOTOPIC CORNEAL ALLOGRAFT SURVIVAL

A total of 50 corneas from B10.D2 mice were grafted orthotopically onto 50 BALB/c mice, of which 30 were randomized to receive topical sTNFR-I treatment and 20 to receive vehicle-only treatment. As shown in Figure 1A, corneal transplants treated with vehicle displayed opacity scores significantly higher than those treated with sTNFR-I. Kaplan-Meier analysis of survival rates (Figure 1B) revealed rates of only 55.0% by week 4 and 40.0% by week 8 in the vehicle-treated group. In contrast, corneal grafts treated with sTNFR-I exhibited survival rates of 83.3% and 75.8% at weeks 4 and 8, respectively (P = .01).

CORNEAL NV

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.

COMMENT

In the present study, prophylactic administration of topical sTNFR-I enhanced the survival of corneal allografts disparate at multiple minor H antigens. These findings, coupled with the previous observation2 that hosts with
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 minor antigens provides a significantly more formidable immune barrier to corneal graft acceptance than disparity at major histocompatibility complex loci.29 Accordingly, Sonoda and Streilein30 showed that grafts disparate at multiple minor H antigens are rejected at the same rate as fully disparate grafts that have disparity at the level of major histocompatibility complex in addition to minor antigens.

The precise action of TNF-α on the induction and expression of corneal alloimmune mechanisms remains incompletely understood. In the murine orthotopic corneal transplantation model, peak secretion of TNF-α protein in allogeneic grafts is observed 1 to 2 weeks after surgery,8 whereas graft rejection typically occurs at 4 to 5 weeks.28,30 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.8-11 In contrast, Larkin et al12 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 data13 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 transplants31,32 is emphasized by data13 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,11 TNF-α need not effect its chemoattractant 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.6,23,34-36 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.25,37,38 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\(^\text{26}\) in major histocompatibility complex and minor H fully mismatched corneal transplantation, increased gene expression of RANTES and MIP1\(\beta\) 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 MIP1\(\beta\). These ligands, by virtue of binding to the widely expressed CCR1 and CCR5 receptors, serve as critical chemotacticants for antigen-presenting cells and activated CD4\(^+\) T cells.\(^\text{3,7,37,38}\) 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 MIP1\(\beta\) 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 TNFR-I used in these experiments, is theoretically limited. However, several factors should be kept in mind. First, as previous work\(^\text{13,28,31}\) 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\(^\text{20}\) 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-\(\alpha\) in corneal angiogenesis remains poorly understood. Inflammatory mediators have been implicated as critical factors for endothelial cell proliferation and angiogenesis.\(^\text{39,40}\) 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\(^\text{28}\) 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-\(\alpha\) 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.\(^\text{17}\) Although they differ in the number of domains and chemical modification, all forms of sTNFR-I have been shown to neutralize TNF-\(\alpha\) 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.\(^\text{17}\) 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.\(^\text{41,42}\)

Currently available preventive and therapeutic regimens for corneal transplant rejection are associated with significant complications.\(^\text{43}\) 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\(^4\), macrophage depletion\(^5\), deviation of recipient immune systems toward T\(_2\) response\(^6\); and intervention of the function of adhesion molecules, cytokines, or CD4\(^+\) T cells.\(^7\)-\(^9\) Our data indicate that local neutralization of TNF-\(\alpha\) activity also holds promise as an effective modality for suppressing TNF-\(\alpha\)-mediated processes in the context of corneal transplant rejection. 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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REFERENCES


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