# Endogenous TNFα Suppression of Neovascularization in Corneal Stroma in Mice

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**Purpose.** To examine the role of tumor necrosis factor α (TNFα) in stromal neovascularization in injured cornea in vivo and in cytokine-enhanced vessel-like endothelial cell tube formation in vitro.

**Methods.** An in vitro model of angiogenesis was used to examine the roles of TNFα on tube formation by human umbilical vein endothelial cells (HUVECs) cocultured with fibroblasts on induction by transforming growth factor β1 (TGFβ1) and vascular endothelial growth factor (VEGF). Central cauterization was used to induce stromal neovascularization in corneas of wild-type (WT) and TNFα-null (*Tnfα*$^{-/-}$) mice. At 7, 14, or 21 days of injury, experimental mice were killed, and the eyes were enucleated and subjected to histologic and immunohistochemical examination and real-time reverse transcription–polymerase chain reaction.

**Results.** HUVECs formed a vessel-like tube structure on the fibroblast feeder layer. Adding TGFβ1, VEGF, or both augmented vessel-like tube formation by HUVECs cocultured with fibroblasts. Adding TNFα (5 ng/mL) completely abolished the formation of tube-like structures despite the presence or absence of TGFβ1 or VEGF in coculture. In vivo, cauterization of the central cornea induced the formation of CD31$^{+}$ new vessels surrounding the limbus in WT mice. More prominent central stromal neovascularization accompanied by increased expression of TGFβ1 and VEGF was found in *Tnfα*$^{-/-}$ mice compared with WT mice.

**Conclusions.** In addition to inhibiting TGFβ1 and VEGF expression by fibroblasts, endogenous TNFα may counter the induction effects of TGFβ1 and VEGF on vascular endothelial cells and may block neovascularization. (*Invest Ophthalmol Vis Sci.* 2007;48:3051–3055) DOI:10.1167/iovs.06-1083

Cornea is an avascular tissue and must remain transparent to refract light properly. Neovascularization in cornea resulting from various inflammatory disorders such as trauma, microbial infection, alkali burn, and limbal stem cell deficiency can impair vision. Cytokines and growth factors orchestrate cell behaviors in the development of corneal neovascularization.[1,2] Vascular endothelial growth factor (VEGF) and transforming growth factor β (TGFβ) are two major cytokines involved in injury-induced neovascularization.[3–6] These cytokines are upregulated in corneal stroma (inflammatory cells and resident corneal cells) during wound healing and inflammatory disorders.[7]

Tumor necrosis factor α (TNFα) is a pleiotropic proinflammatory cytokine.[8] However, the role of TNFα in the development of neovascularization in injured tissues remains largely elusive because results of experiments that examined the roles of TNFα on the pathogenesis of different fibrotic and inflammatory disorders were controversial. For example, it has been shown that the suppression of TNFα by the administration of neutralizing antibody yields a favorable clinical outcome by reducing inflammation.[9,10] This observation is substantiated by the finding that the ablation of TNFα-receptor (*Tnfr*$^{-/-}$) in mice is beneficial in pulmonary fibrosis caused by asbestos.[11] Studies using TNFα-null (*Tnfα*$^{-/-}$) and *Tnfr*$^{-/-}$ mice demonstrated more severe bleomycin-induced pulmonary fibrosis than did wild-type (WT) mice. It has also been reported that the overexpression of TNFα suppresses such bleomycin-induced pulmonary fibrosis.[12–14] These findings imply that TNFα signaling may have a role in modulating inflammatory responses and fibrosis. Therefore, adding TNFα may have beneficial effects on reducing certain types of pathogenic fibrosis while producing adverse effects on others. The pleiotropic roles of TNFα on various pathogenic disorders are further substantiated by the results of studies with collagen-induced arthritis (CIA) in *Tnfα*$^{-/-}$ mice that exhibited some reduction in the clinical parameters of CIA and, on histologic examination, significantly more normal joints. However, severe disease was evident in 54% of arthritic *Tnfα*$^{-/-}$ joints. Interestingly, collagen-immunized *Tnfα*$^{-/-}$ mice developed lymphadenopathy and splenomegaly.[15]

We previously reported that endogenous TNFα could subdue TGFβ1-mediated tissue damage by alkali burn to the ocular surface, as exemplified by the greater severity of tissue damage in *Tnfα*$^{-/-}$ than in WT mice. The tissue damage caused by alkali burn in *Tnfα*$^{-/-}$ mice was accompanied by excess inflammation, myofibroblast generation, and marked neovascularization.[16] Results of our previous studies of *Smad7* gene transfer[17] to alkali-burned cornea and bone marrow transplantation from WT mice to *Tnfα*$^{-/-}$ mice and in vitro coculture experiments revealed that macrophage-derived TNFα could counteract the effect of TGFβ on fibrotic or inflammatory reaction of the alkali-burned cornea.[16,17] However, in our previous study, an alkali burn with sodium hydroxide eyedrop damaged large areas of ocular tissue, including cornea, limbus, and bulbar conjunctiva. Thus, healed corneal surfaces were covered by conjunctival epithelium in WT and *Tnfα*$^{-/-}$ mice.[7,16,17] Therefore, it remains unknown whether such marked corneal neovascularization observed in *Tnfα*$^{-/-}$ mice is associated with an invasion of conjunctival epithelium into the affected cornea or by a lack of TNFα alone. In the present study, to uncover the role of endogenous TNFα in the development of corneal neovascularization, we compared in vivo neovascularization by

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**TABLE 1.** Sequences of Primers and Probes

| Transcript | Sequence |
|---|---|
| mTGFβ1 | F: 5′-gca aca tgt gga act cta cca gaa-3′ |
| | R: 5′-gac gtc aaa aga cag cca ctc-3′ |
| | P: 5′-acc ttg gta acc ggc tgc tga ccc-3′ |
| mVEGF | F: 5′-agc gga gaa agc att tgt ttg-3′ |
| | R: 5′-caa cgc gag tct gtg ttt ttg-3′ |
| | P: 5′-cca aga tcc gca gac gtg taa atg ttc c-3 |

F, forward primer; R, reverse primer; P, probe.

cauterization in WT and *Tnfa*$^{-/-}$ mice and also examined the effects of TNFα on neovascularization using an in vitro model of cultured vascular endothelial cells. Our data indicated that TNFα might directly block neovascularization while it suppressed TGFβ1 and VEGF expression by fibroblasts in situ.

## MATERIALS AND METHODS

Experiments were approved by the DNA Recombination Experiment Committee and the Animal Care and Use Committee of Wakayama Medical University and were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

### Effects of TNFα and TGFβ1 on VEGF Expression in Ocular Fibroblasts

Real-time reverse transcription–polymerase chain reaction (RT-PCR) with TaqMan probes was used to examine the effect of TNFα on VEGF expression by corneal fibroblasts using protocols previously described.[16] Corneal stromal cells derived from explant outgrowth of 2-day-old mice were obtained. Confluent cells were treated with human recombinant TGFβ1 (2 ng/mL; R&D Systems, Minneapolis, MN) or combination TGFβ1 (2 ng/mL) and TNFα (5 or 10 ng/mL; R&D Systems) for 24 hours Six wells were used for each culture condition. Total RNA extracted was subjected to real-time RT-PCR analysis for VEGF mRNA (Table 1). Data were analyzed by unpaired *t*-test.

### In Vitro Coculture Experiment

A commercial coculture system of human vascular endothelial cells (HUVECs) and fibroblasts (NV kit; Kurabo, Tokyo, Japan) was used to examine effects of cytokines on tube-like structure formation. Using this coculture of HUVECs and fibroblasts, we examined the effect of TNFα (5 ng/mL; R&D Systems) on TGFβ1 (0.5 or 1 ng/mL) and VEGF-A (10 ng/mL; Kurabo) stimulation of vessel-like tube formation according to the protocol provided by the manufacturer. Tube tissue was detected by immunostaining with anti-CD31, an endothelium marker. Immune reactivity was visualized by diaminobenzidine color reaction, as previously reported.[7]

### Induction of Stromal Neovascularization by Central Corneal Cauterization in Mice

*Tnfa*$^{-/-}$ mouse in C57BL/6 genetic background was a generous gift from H. Tsutsui (Hyogo Medical University, Hyogo, Japan).[18] Corneal neovascularization from the limbal vessels was induced by central corneal cauterization in one eye of individual WT or *Tnfa*$^{-/-}$ mice using a disposable tool (Optemp; Mod-Tronic Instruments; Brampton, ON, Canada). Experimental mice were killed after 7, 14, or 21 days of injury. Eyes were then enucleated and subjected to cryosection for immunohistochemistry or extraction of total RNA. Ten WT and 10 *Tnfa*$^{-/-}$ corneas were used for histologic examination at each time point. The same numbers of corneas were used for the preparation of total RNA.

### Immunohistochemistry

Immunohistochemical examination was performed to detect stromal neovascularization with anti-CD31 and to measure the production of TGFβ1 and VEGF with respective antibodies. Cryosections (7 μm) were fixed in cold acetone and processed for immunohistochemical examination, as previously reported.[19,20] The following antibodies were diluted in PBS: rat monoclonal anti–CD31 (PECAM) antibody, rabbit polyclonal anti–TGFβ1 antibody, and rabbit polyclonal anti–VEGF antibody (all 1:100 in phosphate-buffered saline, Santa Cruz Biotechnology, Santa Cruz, CA). After fluorescein-conjugated secondary antibody reaction and DAPI nuclear counterstaining, the specimens were observed under a fluorescent microscope. Negative control staining was performed by omission of primary antibodies, which did not yield specific staining (data not shown).

### Detection of mRNAs of TGFβ1 and VEGF in Burned Corneas

Total RNA extracted from two corneas was subjected to analysis of TGFβ1 and VEGF mRNA by real time RT-PCR. Average values from five specimens (10 corneas) at each time point were analyzed by unpaired *t*-test using procedures previously reported (Table 1).[16,17]

## RESULTS

### Effects of TGFβ1 and TNFα on VEGF Expression by Cultured Fibroblasts

Healing of injured corneas is often complicated by neovascularization with the upregulation of VEGF, a major angiogenic factor in physiological and pathologic conditions. Expression of VEGF might be modulated by various cytokines, such as TGFβ1. In the present study, stromal fibroblast cultures were used to examine whether TNFα affects the expression of VEGF. Adding 2 ng/mL recombinant TGFβ1 caused a twofold increase of VEGF mRNA expression by cultured cornea fibroblasts. Upregulation of VEGF mRNA by TGFβ1 was abolished by the addition of TNFα (5 and 10 ng/mL) to the medium (Fig. 1).

### Effects of TGFβ1 and TNFα on Formation of Vessel-like Structure by HUVECs In Vitro

As shown in Figure 1, TNFα antagonized the effects of TGFβ1 on VEGF expression by cultured fibroblasts. Thus, it is likely

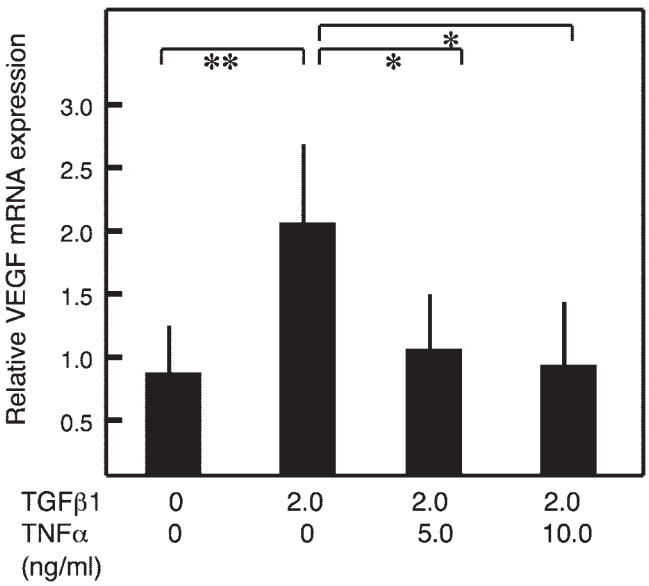


**FIGURE 1.** VEGF expression in mouse corneal fibroblast culture in the presence of TGFβ1, TNFα, or both was examined by TaqMan real-time RT-PCR. TGFβ1 (2 ng/mL) upregulated VEGF mRNA expression, and this was counteracted by the further addition of 5 or 10 ng/mL TNFα. Bar, SD. $^{*}P < 0.05$. $^{**}P < 0.01$, respectively.

that TNFα may suppress the vascularization promoted by TGFβ1. An in vitro model of fibroblasts and HUVEC cocultures was used to examine this possibility. Dense CD31 immunoreactivity was detected in tissue where HUVECs formed a vessel-like tube structure. Without exogenous ligands, HUVECs grown on the fibroblast feeder layer formed a vessel-like tube tissue. Adding TGFβ1 (0.5 ng/mL and 1 ng/mL) promoted the formation of tube-like tissue (data not shown). Adding 5 ng/mL TNFα abolished the formation of a vessel-like tube structure by HUVECs cocultured with fibroblasts, despite the presence and absence of TGFβ1 (Fig. 2). Similarly, adding TNFα abolished the formation of a vessel-like structure in the presence of VEGF-A alone and VEGF-A combined with TGFβ1 (Fig. 3).

## In Vivo Stromal Neovascularization in Cauterized Corneas

To elucidate the possible roles of TNFα on neovascularization after corneal injury, WT and *Tnfα*$^{-/-}$ mice were subjected to corneal cauterization, as described in Materials and Methods. In WT mouse corneas, CD31-labeled neovascularization was not detected at day 7 of cauterization (Fig. 4A). At day 14 (Fig. 4B) and day 21 (Fig. 4C) of cauterization, a few new vessels were seen in peripheral corneal stroma adjacent to the limbus. On the other hand, in *Tnfα*$^{-/-}$ mice, stromal neovascularization was readily detectable as early as day 7 (Fig. 4D) and then increased at day 14 (Fig. 4E). Dense CD31 immunoreactivity was detected in the central corneal stroma of a *Tnfα*$^{-/-}$ mouse (Fig. 4F).

## Expression of Cytokines during Stromal Neovascularization

Given the results of this study, it is plausible to hypothesize that TNFα may have a pivotal role in modulating the expression of angiogenic factors, such as TGFβ1 or VEGF during corneal wound healing. Real-time RT-PCR was used to examine this possibility. Results of real-time RT-PCR showed that cauterization in the central cornea caused an increase of TGFβ1 mRNA expression that persisted until day 21 of cauterization in WT and *Tnfα*$^{-/-}$ mice. TGFβ1 mRNA expression was comparable between WT and *Tnfα*$^{-/-}$ corneas at day 7 of injury, and it was more greatly enhanced in *Tnfα*$^{-/-}$ corneas than in WT corneas at day 21 (Fig. 5A). Results of immunohistochemical analysis of TGFβ1 protein were consistent with those of real-time RT-PCR in that TGFβ1 protein was faintly detectable, with a minor peak at day 14 in WT corneal stroma throughout the intervals examined. In *Tnfα*$^{-/-}$ mice, this cytokine was readily observed in stroma at all the time points examined (Fig. 5B).

Expression patterns of VEGF mRNA and protein were similar to those of TGFβ1. Cauterization in central corneas of WT mice induced an increase of VEGF mRNA expression that peaked at day 14 (Fig. 5C) and then declined to a lower level at day 21. In contrast, the elevated VEGF mRNA expression maintained at a higher level at days 14 and 21 in *Tnfα*$^{-/-}$ corneas was comparable to that of WT mice. VEGF protein was not seen at day 7 and was faintly detected in WT corneal stroma at days 14 and 21. In *Tnfα*$^{-/-}$ mice, this cytokine was markedly observed in all the time points (Fig. 5D). VEGF mRNA expression augmented by the lack of TNFα was more prominent than by the lack of TGFβ1.

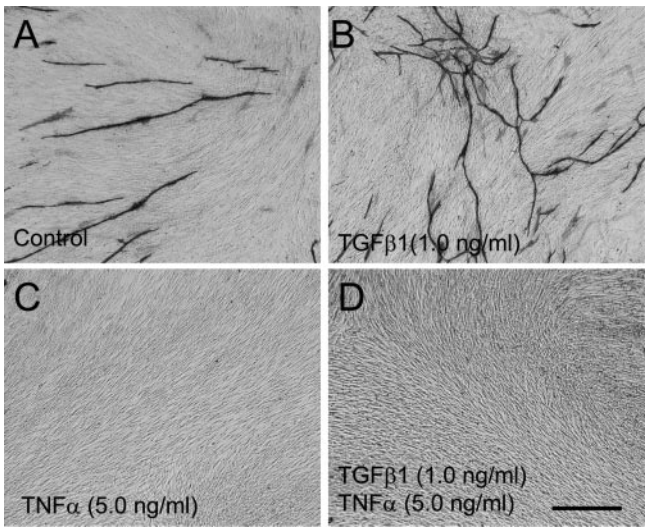


**FIGURE 2.** Vessel-like tube formation as detected by CD31 immunostaining in coculture of fibroblasts and human vascular endothelial cells in the presence of TGFβ1. CD31-immunoreactive vessel-like tissue was seen on the fibroblast feeder layer in the control culture without specific ligands (**A**). (**B**) More dense vessel-like tissue with more frequent branching was seen with 1 ng/mL TGFβ1. (**C**) Adding TNFα (5 ng/mL) blocked the formation of such CD31-labeled structures, even in the presence of TGFβ1 (**D**). Bar, 50 μm.

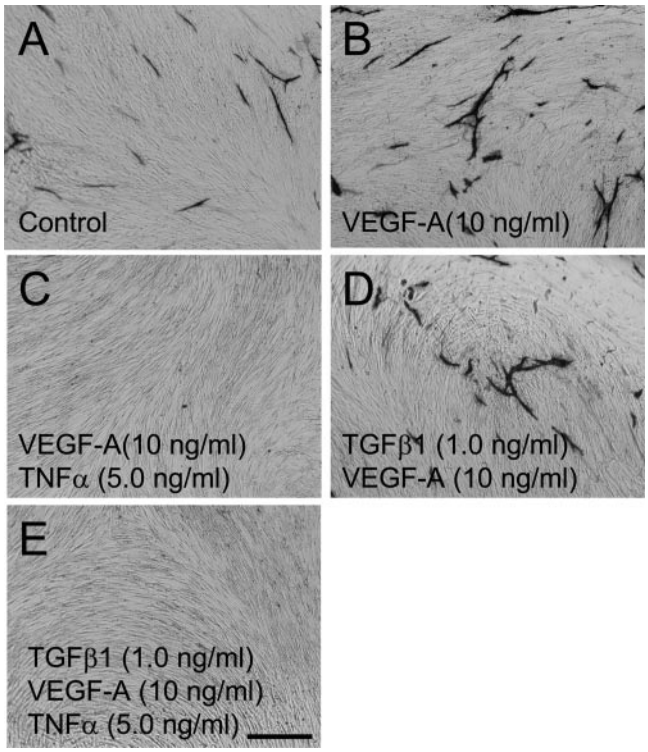


**FIGURE 3.** Vessel-like tube formation was detected by CD31 immunostaining in coculture of fibroblasts and human vascular endothelial cells in the presence of VEGF-A. (**A**) CD31-immunoreactive vessel-like tissue was seen in the control culture without TGFβ1, VEGF, or both. (**B**) More dense vessel-like tissue with extensive branching was seen with 10 ng/mL VEGF-A. (**C**) Adding TNFα blocked the formation of such CD31-labeled structures in the presence of VEGF-A. The formation of vessel-like structures in the presence of TGFβ1 and VEGF-A (**D**) was also abolished by the further addition of TNFα (5 ng/mL; **E**). Bar, 50 μm.

## DISCUSSION

It has been demonstrated that inhibiting TNFα signaling by blocking TNFα receptor did not prevent the development of corneal neovascularization in a rat model.[19] However, the report did not address whether the suppression (or lack) of TNFα signal did not affect, or could promote, corneal neovascularization. We previously reported that *Tnfα*$^{-/-}$ mice experienced more severe fibrosis, inflammation, and neovascular-

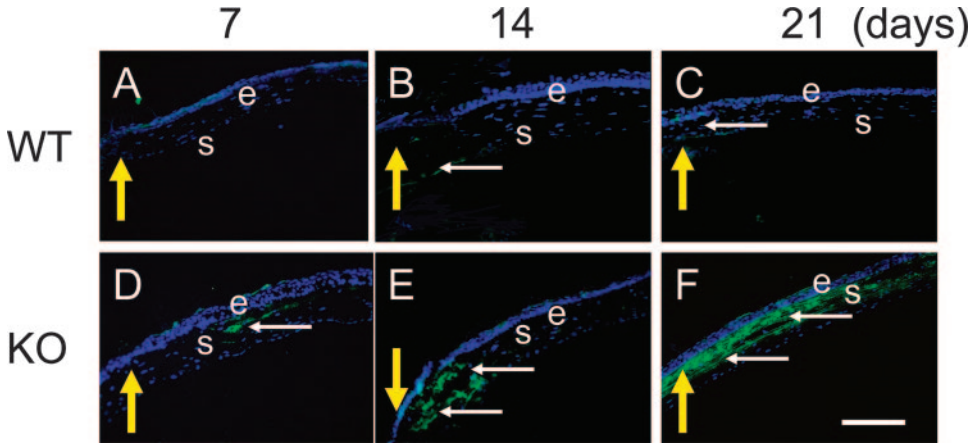


**FIGURE 4.** Immunohistochemical detection of CD31 for stromal neovascularization. CD31 immunoreactivity indicates the presence of neovascularization in the affected stroma. At day 7, no CD31 immunoreactivity was observed in a WT cornea (**A**), and then a few CD31-labeled spots were seen in the peripheral cornea close to the limbus at days 14 (**B**) and 21 (**C**). In *Tnfα*$^{-/-}$ mice, at day 7, CD31-labeled neovascularization was readily detected in corneal stroma (**D**). The density of such neovascularization was increased at days 14 (**E**) and 21 (**F**) in *Tnfα*$^{-/-}$ mice. *White arrows*: CD31-labeled neovascularization; *yellow arrows*: border between cornea and limbus. e, corneal epithelium; s, stroma; DAPI nuclear localization. Bar, 100 μm.

ization in alkali-burned corneas than did WT mice.[16] However, in our previous experimental model of a cornea alkali burn, large areas of ocular surfaces including conjuctiva, limbus, and cornea were injured, and the regenerated ocular surface epithelium was of conjunctival origin associated with neovascularization in the healing cornea. Moreover, in such an alkali burn model, we did not examine whether TNFα could antagonize VEGF-based neovascularization that could be greatly enhanced by TGFβ1. Therefore, we did not know the role of TNFα in corneal neovascularization or its potential in antagonizing TGFβ1 effects in pathogenesis.[20,21]

In the present study, using in vitro coculture of HUVECs and fibroblasts and in vivo *Tnfα*$^{-/-}$ mice, we have demonstrated that TNFα plays a pivotal role in modulating neovascularization. Adding TNFα to the culture medium blocked the tube-like structure formation of HUVECs, even in the presence of TGFβ1 and VEGF. Our data demonstrated that TNFα can directly counteract VEGF action on tube-like structure formation and can suppress VEGF upregulation stimulated by TGFβ1. In vivo, central corneal cauterization caused an upregulation of TGFβ1 and VEGF (Fig. 5). Thus, the expression of TNFα might reduce neovascularization by antagonizing the effects of TGFβ1 and VEGF. This suggestion is supported by the observation that *Tnfα*$^{-/-}$ mice exhibited more severe neovascularization than did WT mice after central corneal cauterization.

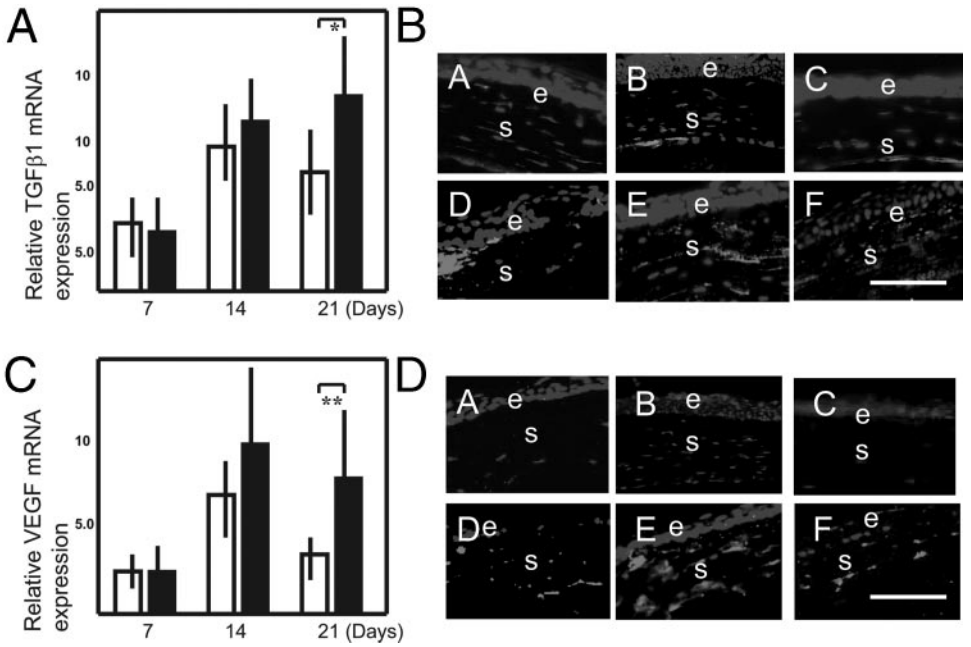


**FIGURE 5.** Expression of angiogenic growth factors in burned corneas. Real-time RT-PCR showed that cauterization in the central cornea caused an increase of TGFβ1 and VEGF mRNAs in the cornea until day 21 in WT and knockout mice. (**A**) Real-time RT-PCR shows the upregulation of TGFβ1 mRNA during the interval examined up to day 21 in WT mice (*open bars*). At day 21, the expression level of TGFβ1 in tissue was significantly higher in *Tnfα*$^{-/-}$ mice (*closed bar*) than in WT mice. (**B**) Immunohistochemistry confirmed the upregulation of TGFβ1 and VEGF revealed by real-time RT-PCR. In WT corneas, TGFβ1 protein was not observed in the stroma at day 7 (**BA**), whereas it was detected in the corneal stroma at days 14 (**BB**) and 21 (**BC**). Overall protein level of TGFβ1 in the stroma was more marked in *Tnfα*$^{-/-}$ corneas at days 7 (**BD**), 14 (**BE**), and 21 (**BF**). (**C**) Real-time RT-PCR reaction showed the upregulation of VEGF with a peak at day 14 and a decline at 21 days in WT corneas (*open bars*). At days 14 and 21, expression levels of VEGF mRNA in tissue were significantly higher in *Tnfα*$^{-/-}$ corneas (*closed bar*) than in WT corneas. (**D**) Immunohistochemistry shows no VEGF protein expression in WT corneal stroma at day 7 (**DA**), whereas faint expression is seen at days 14 (**DB**) and 21 (**DC**). On the other hand, marked VEGF protein expression is observed in knockout stroma at days 7 (**DD**), 14 (**DE**), and 21 (**DF**). e, corneal epithelium; s, stroma; DAPI nuclear localization. Error bars, SD (**A**, **C**). $^{*}P < 0.05$. $^{**}P < 0.01$. Scale bars, 50 μm (**B**, **D**).

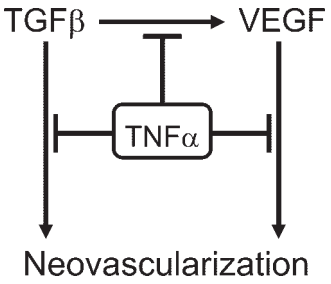


**FIGURE 6.** Hypothetical mechanism of TNFα on corneal neovascularization. TNFα may directly block the angiogenic activity of TGFβ1 and VEGF, and it inhibits the upregulation of TGFβ1 and VEGF by fibroblasts.

Loss of TNFα caused persistent upregulation of VEGF (Fig. 5), which might have accounted for the consequences of augmented neovascularization by central corneal cauterization in *Tnfα*$^{-/-}$ mice. Moreover, in vitro coculture experiments using fibroblast feeder and HUVECs showed that TNFα directly blocked the vessel-like tube formation of HUVECs in the presence of TGFβ1 and VEGF. In corneal fibroblast culture, TNFα blocked the upregulation of TGFβ1 VEGF, also contributing to the antiangiogenic effects of TNFα. Thus, the antiangiogenic effect of endogenous TNFα can be explained in part by a direct counteraction of TNFα against angiogenic reaction promoted by TGFβ1 and VEGF in addition to the suppression of upregulation of such angiogenic growth factors in vivo (Fig. 6).

Many investigators have reported pleiotropic effects of TNFα on various forms of pathogenesis. For example, systemic inflammatory diseases, such as rheumatoid arthritis, can be benefited by the administration of neutralizing anti–TNFα antibody, suggesting that reduction in the local TNFα level is favorable for suppressing inflammation.[22] Such anti–TNFα antibody neutralization is effective in treating bleomycin-induced pulmonary fibrosis.[14] On the other hand, experimental arthritis or pulmonary fibrosis in mice is reportedly more severe in *Tnfα*$^{-/-}$ mice than in WT mice.[13,15] These findings indicate that partial reduction of TNFα and total loss of cytokine exhibit different actions to cells that are implicated in the disease process. It had been suggested that infliximab, a neutralizing antibody against TNFα used clinically for the treatment of rheumatoid arthritis, might also be effective in the treatment of inflammatory ocular diseases.[22] However, our present data implicate that the use of infliximab in treating ocular inflammation involving neovascularization may produce adverse effects.

Taken together, our present findings and reports by other investigators indicate that TNFα is a two-edged sword in modulating the pathogenesis of various diseases characterized by inflammation and fibrosis. The presence of TNFα signaling can alleviate or worsen the diseases. TNFα activates a complicated signaling network that includes c-Jun N-terminal kinase (JNK) and nuclear factor-κB (NF-κB); for a review, see Wajant et al.[8] JNK and NF-κB signals further activate various pathways, such as activator protein-1 or Smads, by inducing Smad7.[8] However, it has been well shown that signaling through TNFα receptor pathways counteract the TGFβ signal at multiple steps, providing a plausible explanation of our observations. Further studies are needed to delineate the details of signaling cross-talk among soluble factors involving TNFα.

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