Impaired Angiogenic Response in the Cornea of Mice Lacking Tenascin C

Takayoshi Sumioka, Noribito Fujita, Ai Kitano, Yuka Okada, and Shizuya Saika

PURPOSE. This study investigated the effects of loss of tenasin C (TNC) in the development of neovascularization in a corneal stroma in mice. Cell culture study was also conducted to clarify the roles of TNC in the expression of vascular endothelial growth factor (VEGF) and transforming growth factor (TGF)β1 in fibroblasts and macrophages.

METHODS. Ocular fibroblasts and macrophages from wild-type (WT) and TNC-null (KO) mice were used to study the role of TNC in the expression of VEGF and TGFβ1. The effects of the absence of TNC on angiogenic gene expression, inflammatory cell invasion, and cornea neovascularization in the corneal stroma were then evaluated after cauterization of the center of the cornea in mice. Histologic, immunohistochemical, and mRNA expression analyses were performed.

RESULTS. Absence of TNC suppressed expression of VEGF and counteracted upregulation of TGFβ1 by exogenous TGFβ1 in ocular fibroblast culture. Such effects of the absence of TNC were not observed in cultured macrophages. Absence of TNC attenuated expression of both VEGF and TGFβ1 mRNA as well as neovascularization into the stroma after cauterization at the center of the cornea in mice. Absence of TNC suppressed macrophages, but not neutrophils, invading the cauterized cornea.

CONCLUSIONS. TNC is involved in angiogenic gene expression in ocular fibroblasts in vitro and in vivo and is required for macrophage invasion and neovascularization of injured corneal stroma. (Invest Ophthalmol Vis Sci. 2011;52:2462–2467) DOI: 10.1167/iovs.10-5750

The cornea is specialized avascular and transparent tissue that refracts light. Trauma, infection, extensive ocular damage, or pathologic limbic stem deficiency induces neovascularization and inflammatory disorders that can potentially impair vision. Neoangiogenesis in an injured cornea is regulated in a complex way by various growth factors that plays critical roles in pro-fibrogenic and pro-inflammatory reactions.1–5 The major cytokine involved in injury-induced neovascularization includes vascular endothelial growth factor (VEGF).6–9 However, cell behaviors are also thought to be modulated by the scaffold of extracellular matrix.10

Tenasin-C (TNC) is one of the wound healing-related matrix macromolecules that is usually transiently upregulated in an injured tissue like fibronectin. It is a disulphide-bonded hexamer of a component composed of subunits with molecular weights in the range of 120–300 kDa. Tenasin-C is also abundantly detected at the invasive margin of cancer, suggesting its role at the progression/acquisition of invasive characteristics by cancer cells.11,12 TNC is reportedly involved in neovascularization in a tissue and serum TNC level increases in angiogenesis in lung cancer.13–16 The expression patterns of TNC in diseased corneas has been reported.17–19 These reports suggest that TNC is expressed in response to stimuli for tissue repair such as fibronectin.20–22 However, the regulation of neovascularization by TNC in the healing process in cornea remains to be elucidated. To address this question in the present study, we used TNC-null (KO) mice to show that the absence of tenasin C in cultured ocular fibroblasts counteracted the acceleration of expression of angiogenic components and that cauterization-induced neovascularization in corneal stroma is attenuated by the suppression of expression of angiogenic growth factors by mice lacking TNC expression.

MATERIALS AND METHODS

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

VEGF Expression in TNC-Deficient Ocular Fibroblasts and Macrophages

To examine the effect of endogenous TNC on VEGF expression in ocular fibroblasts, we conducted a real-time reverse transcription-polymerase chain reaction (RT-PCR) using the previously reported TaqMan probes.3,4 The cells were obtained from C57BL/6 wild-type (WT) and TNC-KO mice as previously reported with a minor modification.23 We enucleated eyeballs from postnatal day 2 mice after CO2 asphyxiation and obtained eyeshells by removing intraocular tissues. The tissues were minced and explanted for the outgrowth of ocular fibroblasts. These cells were positive for vimentin and collagen type I (data not shown). The cells were grown to confluence in a 60 mm culture dish and were then treated with serum-free medium for 24 hours, followed by incubation in serum-free medium or with transforming growth factor (TGF)β1 at 1.0 ng/ml for 24 hours. Six wells were prepared for each culture condition. RNA was extracted and processed for real-time RT-PCR for mRNAs of VEGF and TGFβ1. Data were analyzed by unpaired t-test.

Mouse macrophages were obtained from the peritoneal cavity as previously reported,24 and allowed to adhere to 60-mm plastic dishes for 24 hours. The cells were then treated with serum-free medium for 24 hours, followed by incubation in serum-free medium or with TGFβ1 at 1.0 ng/ml for 24 hours. RNA was extracted and processed for real-time RT-PCR for mRNAs of VEGF and TGFβ1. Data were analyzed by unpaired t-test.

From the Department of Ophthalmology, Wakayama Medical University, 811-1 Kimiidera, Wakayama, 641-0012, Japan.

Supported by a grant from the Ministry of Education, Science, Sports and Culture of Japan (C19592036 to SS, C40433362 to TS), 19 Wakayama Medical Award for Young Researchers Mitsui Life Social Welfare Foundation, Mochida Memorial Foundation, Takeda Science Foundation and Uehara Foundation (to S. S.).

Submitted for publication April 21, 2010; revised September 6 and 14 and October 6 and 13, 2010; accepted October 14, 2010.

Disclosure: T Sumioka, None; N Fujita, None; A Kitano, None; Y Okada, None; S Saika, None

Corresponding author: Takayoshi Sumioka, MD, PhD, Department of Ophthalmology, Wakayama Medical University, 811-1 Kimiidera, Wakayama, 641-0012, Japan; sumioka@wakayama-med.ac.jp.

Copyright 2011 The Association for Research in Vision and Ophthalmology, Inc.
Induction of Stromal Neovascularization by Cauterization of the Central Cornea in Mice

We then performed an in vivo experiment using WT or KO mice. There were no differences in histologic findings in the cornea between WT and KO mice (data not shown). Corneal neovascularization from the limbal vessels was induced by cauterization of the central cornea of the eye using a disposable tool (OPTEMP; Alcon, Fort Worth, TX) as previously reported.8 One eye in each WT and KO mouse (n = 15 of each genotype) was treated and mice were killed on days 3, 7, and 14. The eye was then enucleated and processed for cryo (n = 12 in each genotype) and paraffin sectioning (n = 3 in each genotype). Corneas of each genotype were examined histologically and immunohistochemically.

To examine the expression of angiogenic growth factors and inflammatory cell markers in vivo, centrally cauterized corneas (n = 6 in each of WT or KO group) were excised on days 3 and 7. Total RNA was extracted from cauterized tissue and processed for real-time RT-PCR for VEGF, TGFβ1, F4/80 macrophage antigen, and myeloperoxidase (MPO) as previously reported. Primers for mRNAs of TGFβ1 (Mm03024053 mL), F4/80 (Mm00802524 mL), and MPO (Mm01298422 gL) were purchased from Applied Biosystems (TaqMan; Foster City, CA). Data were statistically analyzed by employing ANOVA.

**Immunohistochemistry**

Immunohistochemistry was performed as previously reported to detect stromal neovascularization and cytokines. New vessels or macrophages were detected by immuno-detection of CD31 antigen, F4/80 (a macrophage marker) or myeloperoxidase (MPO, a marker for a neutrophil leukocytes). Cryosections (7 μm thick) were fixed in cold acetone and processed for immunohistochemistry with rat monoclonal anti-CD31 antibody as previously reported.9,25 Statistical analysis of CD31-labeled neovascularization data were performed by Tukey-Kramer’s test and P < 0.05 was considered significant. Paraffin sections (5 μm thick) were used for detection of F4/80 antigen, MPO, VEGF, and activated form of TGFβ1 as previously reported. Antibody complexation with the peroxidase-conjugated secondary antibody was visualized with 3,3′-diaminobenzidine reaction.

**RESULTS**

Effects of the Absence of TNC on Expression of Angiogenic Cytokines in Fibroblasts and Macrophages

A cell culture experiment was conducted to determine whether endogenous TNC affects the expressions of VEGF and TGFβ1 in the two major wound healing–related cellular components, fibroblasts and macrophages. Adding recombinant TGFβ1 at 1.0 ng/mL did not affect the expression level of VEGF mRNA in both WT and KO fibroblasts. Loss of TNC, however, significantly reduced its expression level in fibroblasts under the conditions with and without exogenous TGFβ1 (Fig. 1a). Adding TGFβ1 to the culture upregulated mRNA expression of TGFβ1 in WT fibroblasts, but not in KO cells. Loss of TNC significantly reduced its expression level in fibroblasts cultured with exogenous TGFβ1 (Fig. 1b). Neither the addition of exogenous TGFβ1 nor the absence of TNC demonstrated significant effects on mRNA expression in cultured macrophages (Figs. 1c, 1d).

Neovascularization in Corneal Stroma

CD31 immunostaining was performed in cryosections. In WT mouse corneas, formation of CD31-labeled neovascularization from the limbus in the corneal stroma was detected in the peripheral cornea on day 3 (Fig. 2a, between black and white...
The length of such neovascularization in corneal stroma peaked on day 7 and then began to decline on day 14. The length of neovascularization was less in KO mice compared with that in WT mice on day 3 and day 7, but not on day 14 (Fig. 2b).

**Expression of Angiogenic Components in Centrally Cauterized Cornea**

Expressions of both VEGF and TGFβ1 mRNA were both significantly lower in KO cornea compared with those in WT cornea on day 3, but such differences were not detected on day 7 (Figs. 3a, 3b).

To detect protein expression of VEGF and TGFβ1, we additionally performed immunohistochemistry. VEGF protein was immuno histochemically detected in tissue. Expression pattern of VEGF was similar to that of F4/80 macrophagic antigen (as shown below), mainly being detected in cells beneath the epithelium. The number of labeled cells observed by immunodetection seemed greater in WT cornea than in KO cornea on day 3 (Figs. 4a, 4b). On days 7 and 14, the protein expression level seemed lower than that on day 3. At these timepoints, the expression level in WT tissues seemed similar to that in KO tissue (Figs. 4c–f). As for the expression pattern of the active form of TGFβ1, activated TGFβ1 was not detected at the immunohistochemical level, although mRNA was detected as described above (Figs. 4g, 4h).

**Expression of Inflammatory Cell Invasion in Centrally Cauterized Cornea**

Immunohistochemistry detected F4/80-labeled macrophages in centrally cauterized corneas (Fig. 5). On day 3, F4/80-positive cells were observed in the full-thickness stroma of the WT central cornea of the cauterization area. At the same timepoint, F4/80-positive cells were detected in the area beneath the epithelium, but not in the deep stroma, in a KO mouse. On days 7 and 14, F4/80-positive macrophages were seen beneath the epithelium in a WT cornea, while there were very few such cells in a KO cornea (Fig. 5). These immunohistochemical findings suggest that the absence of TNC attenuated recruitment of macrophages in the injured corneal stroma. A few MPO-positive cells were observed beneath the epithelium in a WT cornea, but not in a KO cornea, on day 3. On days 7 and 14, MPO-labeled cells were no longer detected in WT and KO tissues (Fig. 6).

To quantify the invasion of macrophages and neutrophiles in tissue we further conducted real-time RT-PCR to clarify mRNA expressions of F4/80 and MPO, as well as mRNA expressions of VEGF and TGFβ1. Expression of F4/80 was suppressed by the loss of TNC on day 3, but not on day 7 (Fig. 7a). Expression of MPO was not significantly affected by the loss of TNC (Fig. 7b).
DISCUSSION

The present in vitro experiments first showed that endogenous TNC is required for angiogenic gene expression in cultured ocular fibroblasts and the development of neovascularization in mouse corneal stroma.

The loss of TNC in cultured fibroblasts indeed suppresses expression of major angiogenic cytokines (i.e., VEGF and TGFβ1). Although it could be postulated that binding of TNC to its specific receptors such as integrins activates cytoplasmic signaling that facilitates crosstalk between TGFβ-derived signals, further detailed study is to be conducted to uncover the mechanism underlying the phenomenon. In macrophages expression of VEGF and TGFβ1 were not markedly affected by the loss of TNC in vitro.

Our real-time RT-PCR then showed that loss of TNC attenuates mRNA expression of angiogenic growth factors (i.e., VEGF and TGFβ1) and suppresses injury-induced neovascularization in mouse corneal stroma on day 3. Macrophages are known to be one of the major components expressing angiogenic cytokines/growth factors in an injured tissue. Our immunohistochemistry suggested that invasion of macrophages in the cauterized corneal stroma might be attenuated by the loss of TNC. This hypothesis was confirmed by the analysis of mRNA expression; expression of F4/80 mRNA was suppressed by the loss of TNC on day 3, but not on day 7. Although expression of angiogenic growth factors (i.e., VEGF and TGFβ1) in a cultured macrophage is not significantly affected by the loss of TNC in vitro, the lower number of macrophages in the stroma of KO mice on day 3 could explain the decreased TGFβ1 and VEGF in expression levels centrally cauterized cornea specimens from KO mice. Immunohistochemical detection of VEGF protein further supports this theory; localization of VEGF-labeled cells was similar to that of macrophages. TGFβ1 protein expression might be below the level of immunohistochemical detection, although real-time RT-PCR clearly showed greater expression in WT tissue. There was no differ-

**FIGURE 4.** Immunohistochemical detection of angiogenic growth factors in centrally-cauterized cornea. Expression of VEGF protein was detected in the cells beneath the epithelium. The number of labeled cells observed by immunodetection seemed greater in WT cornea (a) than in KO cornea (b) on day 3. On days 7 and 14, the protein expression level seemed less than that on day 3. At these timepoints the expression levels in WT tissues seemed similar to those in KO tissue (c–f). There was no apparent TGFβ1 protein immunoreactivity on day 3 (g, h) or at the other timepoints (not shown) in either WT or KO cornea. Bar, 50 μm.

**FIGURE 5.** Immunohistochemical detection of F4/80-labeled cells (i.e., macrophages in the tissue). (A) On day 3, F4/80-positive cells were observed in the full-thickness stroma in the central cauterization area of the WT cornea. (B) At the same timepoint, F4/80-positive cells were detected in the area beneath the epithelium, but not in the deep stroma, in a KO mouse. On days 7 and 14, F4/80-positive macrophages were seen beneath the epithelium in a WT cornea (C, E), while such cells were very few in KO cornea (D, F). Arrows: F4/80-labeled macrophages.
ence in the expression level of MPO neutrophil between WT and KO corneas on either day 3 or day 7, although immunohistochemistry detected a few neutrophil in WT cornea on day 3. Both TGFβ1 and VEGF are chemoattractants for macrophages. Because expression of TGFβ1 and VEGF in cultured ocular fibroblasts was suppressed in the absence of TNC, initiation of macrophage invasion by resident cells in the corneal stroma might be attenuated in KO tissue on tissue cauterization.

TNC is reportedly involved in the persistence of inflammation in various tissue. For example, TNC is expressed in areas of inflammation and tissue damage in inflamed rheumatoid joints. Furthermore, TNC-deficient mice show rapid resolution of joint inflammation. Another report showed that TNC may play a critical role in regulating traffic of macrophages cells in human malignant tumor.26–28 Angiogenic effects similar to those of TNC were reported by another extracellular matrix component, osteopontin; absence of osteopontin impairs the angiogenic responses by myocardium or cornea, as well as the process of tumor progression. Osteopontin upregulates tumor necrosis factor, interleukin (IL)-1β, -6, and -8 in association with p38 phosphorylation in human macrophages.29

Roles of TNC in neovascularization were also investigated in other fields; in a malignant brain tumor, glioblastoma, perivascular deposition of TNC plays a role in angiogenesis and tumor cell proliferation.30 Inflammation, neovascularization, and subsequent tissue fibrosis are key phenomena occurring in tissues after inflammatory diseases and in the healing process post-injury.4,29,31,32 TNC is reported to be involved in the persistence of tissue inflammation in other tissues in addition to its role in the development of neovascularization. For example, zymosan-induced synovitis (joint inflammation) is less severe in KO mice than in WT mice.53 TNC utilizes specific integrin receptors to promote cell reactions against external stimuli. Our unpublished data from immunohistochemical analysis of healing corneas from tenascin C-deficient mice and wild type C57/BL6 mice did not show any difference in alpha v or beta 6 subunit expression in stromal cells.

In conclusion, endogenous TNC modulates the expression of angiogenic cytokines and is involved in the development of corneal neovascularization. To overcome injury induced corneal neovascularization, a better understanding is still required of the complex mechanism modulating this response.

![Figure 6](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932973/)

**Figure 6.** Immunohistochemical detection of myeloperoxidase-labeled (MPO) cells (i.e., neutrophils in the tissue). A few MPO-positive cells were observed beneath the epithelium in WT cornea (a), but not in KO cornea (b), on day 3. On days 7 and 14, MPO-labeled cells were no longer detected in WT or KO tissues (c–f). Bar, 50 μm.

![Figure 7](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932973/)

**Figure 7.** Relative mRNA expression levels of F4/80 antigen and myeloperoxidase (MPO) in the affected cornea. Expression of F4/80 (a), a marker for macrophages, was also lower in KO cornea than in WT cornea on day 3. There was no difference in the expression levels of myeloperoxidase (MPO), a marker for a polymorphonuclear leukocytes, between WT and KO corneas on days 3 and 7 (b). P < 0.05 by ANOVA test.
References


