Inhibition of Inflammatory Corneal Angiogenesis by TNP-470

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PURPOSE. To determine the efficacy of the angiogenic inhibitor TNP-470 on inflammatory corneal neovascularization. Topical and systemic delivery of the drug were investigated in a murine model as well as inhibition of endothelial cell proliferation in vitro and in vivo.

METHODS. The effect of TNP-470 on VEGF- and bFGF-stimulated bovine capillary endothelial (BCE) cell proliferation was evaluated in vitro. Corneal neovascularization was induced in vivo by mechanical debridement of the corneal and limbal epithelium with 0.15 M NaOH on C57BL6 mice. TNP-470 was administered systemically at 30 mg/kg body weight (BW) every other day or topically three times daily in a concentration of 5 ng/ml dissolved in methylcellulose. Vessel length was investigated on day 7. VEGF protein content in murine corneas was analyzed by ELISA on days 2, 4, and 7 of treatment. A modified bromodeoxyuridine (BrdU) ELISA was used to quantify endothelial cell proliferation.

RESULTS. TNP-470 exerted a dose-dependent inhibition of bFGF- and VEGF-induced endothelial cell proliferation in vitro. Both systemic and topical application of TNP-470 led to a significant reduction of inflammatory corneal neovascularization (P < 1×10-5). BrdU labeling showed that TNP-470 inhibited endothelial cell proliferation. VEGF protein levels were reduced by systemic TNP-470 treatment.

CONCLUSIONS. These results suggest that TNP-470 reduces inflammatory corneal angiogenesis by directly inhibiting endothelial cell proliferation. Topical and systemic treatment with TNP-470 reduces VEGF levels that are responsible for vessel growth during the neovascularization process. (Invest Ophthalmol Vis Sci. 2001;42:2510–2516)

Angiogenesis is an important process in physiological and pathophysiological situations. Neovascularization is a common feature of inflammatory, infectious, and traumatic diseases of the cornea and the limbal stem cell barrier. Neovascularization remains a severely disabling condition, resulting in loss of the immunologic privilege of the cornea and in visual impairment. Although certain forms of ocular neovascularization are susceptible to laser treatment or surgical intervention, corneal neovascularization responds poorly to laser ablation. In various pathologic conditions, recognition of the potential benefits of controlling angiogenesis has led to a search for natural and/or synthetic angiogenesis inhibitors.1–7 Most of these substances have been analyzed using the corneal pocket model for stimulation of corneal angiogenesis with vascular endothelial growth factor (VEGF) or basic fibroblast growth factor (bFGF).

TNP-470 (AGM 1470) is an analogue of fumagillin, identified as an angiogenesis inhibitor by Ingber et al.8 Cultures contaminated with Aspergillus fumigatus showed an inhibition of endothelial cell proliferation due to fumagillin, the antibiotic protein secreted by the fungus. The artificial analogue TNP-470 retains the same antiangiogenic activity but has fewer side effects. TNP-470 has been shown to inhibit tumor growth in human tumor xenografts (e.g., of colon, breast, and pancreatic tumors).9–11 Currently, phase II clinical studies are in progress in solid tumors, including metastatic renal carcinoma, carcinoma of the cervix, melanoma, nerve sheath tumors, and numerous others.12–13 TNP-470 is effective and relatively safe for clinical use. The principal toxicity is neurotoxicity that is reversible and dose dependent. However, the actual molecular targets of TNP-470 are still unknown.

In the treatment of ocular neovascular disease, TNP-470 has recently been shown to be effective in a rat model of laser-induced choroidal neovascularization.14,15 Moreover, inhibitory effects after systemic treatment have also been shown on bFGF-induced angiogenesis in the cornea.16 In the corneal pocket model in mice. It may therefore become clinically useful for a variety of ocular diseases involving neovascularization, such as wound- and inflammation-related corneal angiogenesis with limbal insufficiency, which is still pharmacologically untreatable. In this study, we analyzed the efficacy of TNP-470 as an inhibitor of inflammatory corneal angiogenesis.

MATERIALS AND METHODS

Treatment with TNP-470

TNP-470 was generously supplied by Takeda Inc., Osaka, Japan. One-hundred mg TNP-470 in powder was stabilized with 840 mg betacyclodextran and dissolved in sterile PBS to a final concentration of 3 mg TNP-470/ml. Frozen aliquots were thawed immediately before use.

For systemic application, 30 mg/kg TNP-470 was injected subcutaneously once every other day, as described previously.8 Control animals received equal volumes of the vehicle in subcutaneous injection.

For topical application, TNP-470 was dissolved in sterile hydroxypropylmethylcellulose 2.5% (Gonisol; Iolab, Claremont, CA) and applied at a final concentration of 5 mg/ml three times daily.

Bovine Capillary Endothelial Cell Proliferation Assay

Bovine capillary endothelial (BCE) cells were isolated and maintained as previously described.17 For the proliferation assay, confluent cells between passages 9 and 14 were dispersed with trypsin-EDTA (Gibco BRL, Grand Island, NY). A suspension of 2.5 × 105 cells/ml in DMEM containing 10% bovine calf serum (BCS; HyClone, Logan, UT) and 1% glutamine penicillin-streptomycin (GPS, Irvine Scientific, Santa Ana, CA) was made, and 0.5 ml of this suspension was cultured for 24 hours in gelatin-coated 24-well plates at 37°C in 10% CO2. Twenty-four hours...
later, the medium was replaced with 0.25 ml DMEM containing 5% BCS and 1% GPS, with or without TNP-470 supplementation, at concentrations ranging from 0.005 to 1 ng/ml. The concentration of BCS was maintained at 5%, as incubation without serum for more than 72 hours leads to a significant increase in cell death. Basic fibroblast growth factor (bFGF) was added where applicable after 20 minutes to a final concentration of 1 ng/ml bFGF. In a second set of experiments, both bFGF and VEGF, at various concentrations, were applied together with 500 pg/ml TNP-470. VEGF was used at concentrations ranging from 0 to 8 ng/ml. For negative controls, neither bFGF nor VEGF was added. Seventy-two hours later, the cells were trypsinized and resuspended in a balanced electrolyte solution (Coulter Corp., Hialeah, FL) and counted with a cell counter (model Z1; Coulter Corp.). All experiments were performed at least in triplicate in three independent experiments.

Animals
C57Bl/6 mice, weighing 20 to 25 g, were purchased from Jackson Laboratories (Bar Harbor, ME). All animal experiments followed the guidelines of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Animal Care and Use Committee of Children’s Hospital, Boston. All surgical procedures were performed in animals under general anesthesia (intramuscular administration of 5 mg/kg xylazine hydrochloride and 35 mg/kg ketamine hydrochloride). To monitor systemic side effects of the treatment, body weight, and temperature were measured on every observation day. Animals were held in groups of 10 and fed regular laboratory chow and water ad libitum. A 12-hour day and night cycle was maintained.

Induction of Corneal Neovascularization
Under intramuscular general anesthesia using xylazine (10 mg/kg; Bayer, Leverkusen, Germany) and ketamine hydrochloride (150 mg/kg; Phoenix, St. Joseph, MO) and additional topical application of lidocaine (Alcon, Fort Worth, TX), inflammatory neovascularization was induced by application of 2 μl of 0.15 mM NaOH to the central cornea of each mouse. The mice were randomly divided into three groups that received treatment with vehicle, topical TNP-470, or systemic TNP-470. Each group consisted of 13 animals, unless otherwise specified (26 corneas in total per group). The corneal epithelium was subsequently scraped off with a blunt von Graefe’s knife. The limbal areas were gently massaged over 360° for 3 minutes. To prevent infection, eyes were treated with antibiotic ointment (3.5 IE/mg neomycin sulfate, 0.3 IE/mg bacitracin, and 7.5 IE/mg polymyxin B sulfate; Polyspectran: Alcon, Friedberg, Germany) after surgery. Each set of experiments was repeated three times.

Visualization and Quantification of Corneal Neovascularization
One week after epithelial debridement all 13 control and systemically and topically TNPTreated mice were perfused systemically with FITC-coupled Concanavalin A (ConA) lectin, which binds to the vascular endothelial cells and permits visualization of corneal neovascularization. After induction of deep anesthesia, the chest was carefully opened, and a 20-gauge canula was placed into the left ventricle. Physiological pressure of 80 mm Hg was maintained while the heart was pumping. Fixation was then achieved by perfusion with 1% formaldehyde and 0.5% glutaraldehyde, maintaining the 80-mm Hg pressure and using a total volume of 200 ml/kg BW over approximately 5 minutes. After inhibition of nonspecific binding with 1% albumin in PBS (total volume 100 ml/kg BW), the perfusion was continued with a balanced electrolyte solution (Coulter Corp., Hialeah, FL) and counted with a cell counter (model Z1; Coulter Corp.). All experiments were performed at least in triplicate in three independent experiments.

For measurement of vessel length, the limbus was examined during fluorescence microscopy as the line between the iris pigment epithelium and the clear cornea. Measurements of vessel length were taken at two distinct points in each quadrant. Quantification of the vascularized area was determined with the following formula: \( x(r^2 - r - L^2) \), an ellipse formula, where \( r \) is 1.6 mm and \( L \) is the measured length of the vessel. For a second, independent quantification, images of the perfused corneas were captured using a charge-coupled device (CCD) camera (model CD-350; Dage-MIT, Inc., Michigan City, IN) attached to a microscope (MZ FLIII; Leica Microsystems Inc., Deerfield, IL). The images were captured on a computer (model G4; Apple, Cupertino, CA) and analyzed (Openlab software; ImproVision, Inc., Lexington, MA). The images were resolved at 624 × 480 pixels and converted to tagged information file format (TIFF) files. The neovascularization was quantified by setting a threshold level of fluorescence, above which only vessels were captured (density slicing). The entire mounted cornea was analyzed to minimize sampling bias. The total surface area of the cornea was outlined, using the innermost vessel of the limbal arcade as the border. The total neovascularization area was then normalized to the total corneal area, and the percentage of the cornea covered by vessels was calculated.

All corneas were photographed with a standardized technique to compare vascular density. Corneal angiogenesis was scored on a graded scale (1+ for minimum response and 4+ for maximum response). The grade level 1+ comprised corneas showing 0 to 4 vertical neovascular vessels per visual field; 2+, 5 to 10 vertical vessels; 3+, 11 to 20 vertical vessels; and 4+, more than 20 vertical vessels. The grading was performed in a masked manner.

Analysis of Cell Proliferation with Bromouridine
One week after epithelial debridement five control animals without TNP-470 treatment and five mice treated with TNP-470 systemically and five treated topically received an intraperitoneal injection of bromouridine (BrdU; Sigma, Munich, Germany) at a volume of 1 ml/100 g BW from a 10-mM solution (50 mg/kg BW). Two hours after the injection, the mice were perfused with FITC-coupled ConA lectin, and their corneas were flatmounted as described earlier. The corneal epithelium was removed gently with ethanol swabs (Johnson & Johnson; New Brunswick, NJ) to facilitate penetration of the antibody. Subsequently, the tissue was permeabilized with 1% Triton X-100 (Sigma) in PBS for 24 hours at 4°C. Nonspecific binding was blocked with 10% goat serum in 1% BSA (Sigma) for another 2 hours. After overnight incubation at 4°C with a primary anti-BrdU antibody (Boehringer Mannheim, Mannheim, Germany) and subsequent washing steps, the secondary antibody was applied for 1 hour at room temperature (Texas red–conjugated goat anti-mouse antibody; Oncogene Research, Cambridge, MA). To ensure specific staining, control specimens were stained without primary antibody. After washing, specimens were viewed with fluorescence microscopy, as described previously.

Quantification of Cell Proliferation with BrdU ELISA
One week after epithelial debridement, seven control animals and seven mice treated with TNP-470 systemically and seven treated topically received an intraperitoneal injection of BrdU (Sigma) at a concentration of 50 mg/kg BW. Two hours after the injection, the animals were killed, and the corneas were dissected and digested in 100 μl trypsin-EDTA (Gibco) containing 1% dispase (Gibco) for 60 minutes at 37°C. The digested cells were transferred into a 96-well plate for 20 minutes, and the plate was centrifuged at 2000g for 20 minutes, for the detached cells to attach to the plate. The amount of incorporated BrdU was assayed with the cell proliferation ELISA (Boehringer) according to the manufacturer’s instructions. The amount of incorporated BrdU was measured by the absorbance at 370 nm.
ELISA for VEGF

Eight control animals, and seven mice treated with TNP-470 systemically and seven treated topically were prepared for VEGF assay as for the BrdU ELISA and killed on days 2, 4, and 7 after treatment. Corneas were dissected and placed in 60 μl lysis buffer (20 mM imidazole HCl, 10 mM KCl, 1 mM MgCl2, 10 mM EGTA, 1% Triton, 10 mM NaF, 1 mM Na molybdate, 1 mM EDTA [pH 6.8]) supplemented with a protease inhibitor cocktail (Boehringer Mannheim, Indianapolis, IN) followed by mechanical homogenization. The lysate was cleared of debris by centrifugation at 14,000 rpm for 15 minutes (4°C), and the supernatant was collected. Total protein was determined with a commercial assay (BCA kit; Bio-Rad, Hercules, CA). VEGF levels were determined by sandwich ELISA, according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN) and normalized to total protein.

Statistical Analysis

To analyze the differences between treated and control eyes, as well as within the treatment groups, an unpaired t-test with two-tailed probability or ANOVA (for multiple comparisons) was used. Results are presented as mean ± SEM.

For each experiment, surgery was performed on all animals in a standardized fashion, and animals were randomized to the different treatment and control groups.

RESULTS

In Vitro Effect of TNP-470 on BCE Cells

Under in vitro conditions, TNP-470 inhibited the proliferation of BCE cells in a dose-dependent manner (Fig. 1A). When BCE cells were stimulated with 1 ng/ml bFGF or 8 ng/ml VEGF, significant inhibition occurred at 0.5 and 1 ng/ml TNP-470 (P < 0.01). Stimulation of BCE proliferation with VEGF in concentrations ranging from 0 to 8 ng/ml was inhibited by TNP-470 at 0.5 and 1.0 ng/ml (P < 0.00001; Fig. 1B). TNP-470 reduced nonstimulated BCE growth by 36.7% and attenuated VEGF-induced proliferation. When BCE cells were costimulated with bFGF (1 ng/ml) and VEGF, TNP-470 was still efficient in inhibiting growth (P < 0.00001; Fig. 1C), with comparable potency in all VEGF concentrations used.

Inhibition of Inflammatory Angiogenesis

As shown in Figure 2, vessel length and vascularized area were significantly reduced by both systemic and topical treatments with TNP-470 on day 7 after corneal scraping. The average vessel length in 25 scraped control corneas, which were measured at eight distinct points each, was 1.06 ± 0.13 mm. The vessel length was reduced by 60.52% (0.41% ± 0.05%) in animals treated with intraperitoneal TNP-470 (30 mg/kg BW) every other day (P < 1 × 10−11). Topical treatment with TNP-470 (5 ng/ml, three times daily) inhibited the vessel-length by 44.64% (0.58% ± 0.24%, P < 1 × 10−7). If calculated with the ellipse formula, the vascularized area was accordingly inhibited by 29% of the total cornea area with systemic treatment and by 22.7% with topical application of TNP-470 (P < 1 × 10−5). Accordingly, with image analysis (density slicing), the reduction in the vascularized area in the TNP-470–treated group was 67.1% in the systemically treated and 37.5% in the topically treated group (Fig. 2B).

Vessel morphology was investigated in flatmounts after lectin staining of the cornea. Whereas control eyes showed a dense capillary network with multiple leukocytes adhering to the vessel walls (Fig. 3), eyes of the TNP-470–injected group had reduced vascular density. Eyes of the control group typically showed sharp-edged tips of the vascular channels. After TNP-470 treatment, endothelial sprouts were only rarely visible. Eight of 10 corneas in the control group were graded 4+ for vascular density. The average estimated density of the TNP-470–treated group for both topical and systemic application was 2+, ranging from 1+ to 3+. In vivo proliferation of endothelial cells was inhibited by TNP-470.

To investigate the effect of TNP-470 local or systemic treatment on endothelial cell proliferation, BrdU labeling was performed on days 2, 4, and 7 after corneal scraping in animals receiving systemic (subcutaneous injection) TNP-470 or control vehicle. Proliferating cells synthesize nucleic acids and thus incorporate BrdU in their DNA. In vivo labeling with BrdU was initially performed at different time intervals. If BrdU was injected for more than 4 hours before death, major parts of the corneal epithelium and a few stromal cells were found to be
positive. With a BrdU incorporation time of 2 hours, vascular endothelial cells sprouting off of actively growing vessels in control animals were almost selectively marked. In TNP-470–treated animals the cell turnover of endothelial cells was slower; hardly any sprouting endothelial cells were detected after BrdU incubation. Most BrdU immunoreactivity was localized at the tips of the vascular sprouts (red). ConA counterstaining showed the inner border of the endothelial cells (green). Original magnification, ×400. BrdU labeling was quantified by cell proliferation ELISA. Compared with untreated corneas, limbal injury caused a significant increase of cell proliferation at all time points (n = 3, P < 0.001). Treatment with TNP-470 reduced cell proliferation by 80.24% on day 2, by 77.9% on day 4, and by 77.0% on day 7 after limbal injury (P < 0.005).

To ensure that topical application of TNP would not delay wound closure after scraping, three corneas in every treatment group were stained with fluorescein once daily and examined under the microscope for integrity of the epithelium. Topical treatment with TNP-470 was started 12 hours after surgery. Re-epithelization of corneas from animals with either topical treatment of TNP or untreated scraped control corneas, showed complete wound closure on day 2 after surgery. Thus, proliferation of the corneal epithelium was not affected in a clinically relevant manner by topical TNP treatment.

Modification of VEGF Expression in Inflammatory Angiogenesis by TNP-470

VEGF was shown to play a key role in the induction of corneal neovascularization in our murine model of inflammation-induced corneal neovascularization. To investigate the ability of systemic treatment with TNP-470 to reduce production of VEGF protein, corneal VEGF levels were assayed by an ELISA.
Two days after limbal injury, corneas of TNP-470–treated animals contained 3.47 ± 1.81 pg VEGF/µg corneal protein, compared with 9.84 ± 1.3 pg VEGF/µg corneal protein in vehicle-treated control animals (n = 3, P < 0.05). On day 4 of treatment, corneas of TNP-470–treated animals contained 3.0 ± 0.05 pg VEGF/µg corneal protein compared with 11.49 ± 1.8 pg VEGF/µg corneal protein in control animals (n = 3, P < 0.0001). Seven days after limbal injury, 14.04 ± 1.76 pg VEGF/µg total corneal protein was detected in control animals. Treatment with TNP-470 resulted in 9.91 ± 1.60 pg VEGF/µg total corneal protein on day 7 (n = 3, P < 0.005; Fig. 5).

DISCUSSION

Corneal neovascularization is a major sight-threatening complication of corneal infections, chemical injury, and keratoplasty. It is characterized by a corneal ingrowth of new vessels originating from the limbus, often accompanied by an inflammatory response. Recently, it was suggested that VEGF plays an important role in this process, because exogenous VEGF stimulates corneal neovascularization and a neutralizing anti-VEGF antibody inhibits it.22 In the present study, topical or systemic treatment with TNP-470, a synthetic analogue of fumagillin, inhibited endothelial cell proliferation, reduced VEGF protein levels, and thus inhibited angiogenesis in a murine model of inflammatory corneal neovascularization.

We found that TNP-470 inhibited bFGF- and VEGF-stimulated BCE cell proliferation in vitro and endothelial cell proliferation in vivo. TNP-470 has been shown primarily to inhibit endothelial cell proliferation, which is important for its binding sites on its receptor. 31 How-ever, by interfering with the bFGF pathway, TNP-470 inhibits cutaneous wound healing, and this effect can be overcome by topical application of bFGF.32 Conversely, in our model, corneal wound closure was the same in control and TNP-470–treated mice, a finding that can be attributed to the differential effect of TNP-470 on specific cell types. Although further studies are needed to address the mechanism of this cell-type-specific selectivity of its actions, our results indicate that topical application of TNP-470 at a certain dose shows minimal effects on corneal epithelial cells and is still effective on vascular endothelial proliferation.

In our model of inflammatory corneal neovascularization, VEGF appears to be a major player.21,35 We have shown that corneal scraping upregulates VEGF with a peak on day 2 after scraping, and that VEGF upregulation correlates with increased leukostasis.34 Leukostasis seems to have a strong effect on the angiogenic response, in that inhibition of the leukocyte–endothelial interaction through blockade of adhesion molecules decreases corneal inflammatory neovascularization.21,35 In the present study, TNP-470 reduced VEGF protein levels in the cornea, thus contributing to the inhibition of the corneal neovascularization process.

To our knowledge, regulation of VEGF by TNP-470 in the cornea has not been previously reported. In a recent report, it was demonstrated that TNP-470 activates the p53 pathway. TNP-470 inhibits MetAP2, resulting in defects in N-terminal processing of proteins, generation of aberrant proteins, and cellular stress, which activates p53 and subsequently p21.26 Elevated p53 activity has been shown to inhibit angiogenesis in vivo by downregulating VEGF expression.35 Thus, TNP-470 may downregulate VEGF directly through p53. Alternatively, TNP-470 was shown to inhibit myristoylation and membrane translocation of nitric oxide (NO) synthase, thus increasing the cellular content of NO synthase and the production of NO.36 NO downregulates VEGF expression both in vivo and in vitro.37

In our model, the decrease of VEGF protein was more prominent on days 2 and 4 of TNP-470 treatment than on day 7. This could indicate that TNP-470 reduces VEGF mRNA in the endothelial cells through the mechanisms described earlier in combination with its effect on endothelial cell proliferation and thus leads to reduced VEGF secretion, but has no direct effect on the inflammatory reaction. The less prominent decrease in VEGF protein content on day 7 may provide indirect evidence for a second pathway for VEGF impact in inflammatory angiogenesis and enhances the significance of the role of inflammatory monocytes in producing VEGF.34 There is another cell type that may contribute to VEGF production in fibroblasts. An elegant study in transgenic mice has demonstrated that VEGF is produced in a healing wound by fibroblasts.38 With the same model, it has been shown that in the microenvironment of a carcinoma, which mimics that of a wound, the production of VEGF in fibroblasts is stimulated by the neoplastic epithelial cells.39

In our model, as the superficial wound healed, the epithelial cell and fibroblast population increased, and the production of VEGF may increase through their interaction. This hypothesis could explain the late rebound in VEGF levels, after the re-epithelialization of the surface wound. Alternatively, the delayed decrease in TNP-470 effectiveness in lowering VEGF levels may suggest the activation of counterregulatory intracellular mechanisms that desensitize the target(s) of TNP-470 to its effect(s). In any case, these findings suggest that early, rather than late, administration of TNP-470 is warranted in a clinical setting to prevent long-term sequelae.

To find doses of TNP-470 suitable for clinical use in ocular disease, we explored the effects of the systemic administration of a dose of 30 mg/kg BW every other day, which is known to be effective in cancer therapy.8,11 This treatment regimen was found to be effective against inflammatory corneal neovascularization. The effects of a single dose of TNP-470 are sustained after 48 hours,16 although the mechanisms that are involved in...
this prolonged action of TNP-470 are not known. TNP-470 is rapidly broken down into six metabolites as early as 6 minutes after intravenous administration, but the antiangiogenic activity of these metabolites has not been investigated. For use in ocular anterior segment disease, a topical application protocol would be preferable. In this regard, in vitro studies have shown a half-maximal cytostatic inhibition of endothelial cell proliferation at 10 pg/ml. After stimulation with VEGF and bFGF, we found that even doses as low as 10 ng are effective in vitro. A topical dose of 5 ng/ml (equivalent to 0.005%), given three times daily, inhibited corneal neovascularization almost as much as systemic application. Individual variability of the response to systemic application was higher in topical application, probably due to the anatomic features of the mouse, which has little or no conjunctival sac and little tolerance for eye drops. However, topical application could make TNP-470 suitable not only for inhibition of corneal neovascularization, but also for fibrovascular diseases, such as pterygium or wound healing after glaucoma filtration surgery.

In conclusion, TNP-470 effectively inhibits endothelial cell proliferation and inflammatory corneal angiogenesis. Because this agent has generally been well tolerated in human clinical trials, it holds promise for the treatment of corneal neovascularization, whether used alone or in combination with other anti-inflammatory agents.

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References


