Angiogenesis Inhibitor TNP-470 Inhibits Murine Cutaneous Wound Healing

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Submitted for publication September 3, 1998

Conclusion. Therapy with TNP-470 induces a significant delay in murine cutaneous wound healing. This effect may be exploited for use in situations where wound healing is excessive and debilitating. Topical application of bFGF can overcome TNP-470-induced wound healing inhibition. © 1999 Academic Press

Key Words: TNP-470; AGM-1470; wound healing; angiogenesis inhibitor; basic fibroblast growth factor (bFGF).

INTRODUCTION

Background. TNP-470 (AGM-1470) is a potent inhibitor of angiogenesis with potential therapeutic applications in neoplastic and angioproliferative diseases. This study evaluated its effect on cutaneous wound healing in a murine dorsal excisional wound model.

Materials and methods. Full-thickness wounds (1.60 cm²) were created on the dorsum of homozygous/ hairless mice (7 to 9 weeks). Wound areas were measured on alternate days for 16 days. Experimental groups consisted of (1) TNP-470 administered in doses of 0.05, 0.5, and 5.0 mg/kg on Days 0, 2, and 4 or Days 0 through 6; (2) TNP-470 (5.0 mg/kg) coadministered with minocycline (4.0 and 10 mg/kg) on Days 0, 2, and 4; and (3) TNP-470 (5.0 mg/kg on Days 0, 2, and 4) coadministered with topical basic fibroblast growth factor (bFGF) 1.0 μg/wound on Days 0, 1, and 2. Hematoxylin and eosin staining was used to compare experimental and control wounds.

Results. TNP-470 administration significantly decreased wound healing in a dose-dependent manner versus controls (P < .05). The 5.0 mg/kg concentration yielded the greatest effect by maintaining an average wound area 20.4% greater than controls and a marked delay in wound healing on H&E staining. Alternate-day dosing was as effective as consecutive day administration. Minocycline did not augment the wound healing inhibition of TNP-470. Coadministration of TNP-470 and bFGF eliminated any rate-altering effect of TNP-470 upon wound healing and resulted in wound areas similar to controls.

1 This research was made possible through a grant from the Jewish Hospital Foundation, Louisville, KY (Grant 970615-04).

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The efficacy of TNP-470 as an angiogenesis inhibitor has been demonstrated experimentally for the treatment of hemangiomas [4], corneal neovascularization [8], and primary tumors and metastatic disease [5,9]. Additionally, it has been demonstrated that the antiangiogenic effects of TNP-470 can be augmented with the addition of minocycline in a murine Lewis lung carcinoma model [10]. It has been speculated that the inhibitory effect of TNP-470 on angiogenesis is due to an inhibition of the actions of basic fibroblast growth factor (bFGF) [8, 11].

The roles of bFGF are well documented in the wound healing process. Basic FGF is a chemoattractant for numerous cell types [12], a potent mitogen for angiogenesis [13], and an activator of wound contraction [14]. Additionally, exogenous administration of bFGF has been shown to increase the rate of wound closure [15]; therefore, inhibition of the effect of bFGF, used with TNP-470, could potentially modulate the wound healing process. We sought to investigate what effects an angiogenesis inhibitor would have on a wound left to heal by secondary intention and whether these effects could be modified with the simultaneous administration of additional agents.

**METHODS**

**TNP-470**

TNP-470 was obtained from TAP Holdings Inc. (Deerfield, IL) and stored dry at −15°C. Prior to subcutaneous injection, TNP-470 was dissolved in 2% ethanol and diluted in 0.9% normal saline. A vehicle control solution was made by the addition of 2% ethanol solution to 0.9% normal saline.

**Animal Housing and Anesthesia**

Male homozygous hairless mice (Jackson Laboratories, Bar Harbor, ME) between the ages of 6 to 8 weeks were housed individually in a controlled environment with normal light-dark cycles and a constant temperature maintained between 78 to 82°C. The mice were acclimated to their environment for 1 week before the study began and were provided water and rodent chow ad libitum. Each mouse was individually anesthetized with inhaled Metofane (Pitman-Moore, Mundelein, IL) and immediately weighed postanesthesia. All animals were handled in accordance with the guidelines established by the University of Louisville Animal Care and Use Committee.

**Surgical Manipulation**

On Day 0 of the study, a full-thickness wound, including the panniculus carnosus, was excised from the dorsum of each mouse. Using a standard template, a 2.0 cm² circular defect was outlined 2.0 cm from the nape of the animal’s neck using a fine-tipped marking pen. The defect was created by elevating the skin and panniculus carnosus in the center of the outlined area using nontoothed forceps, followed by excision of the outlined area using microdissecting scissors. The wounds were continuously covered with both an nonadherent dressing (petrolatum gauze [Kendall-Curity, Mansfield, MA] or Duoderm gel [Convatec, Princeton, NJ]) and an occlusive dressing (Tegaderm, 3M, St. Paul, MN). The wounds were allowed to close by secondary intention.

**Data Acquisition and Analysis**

Each animal was placed on its ventral surface with all limbs extended laterally for wound area measurements: The area of the wound was viewed at a final magnification of 25× using a color video camera (Mintron, OS-70D), Fremont, CA) attached to the eyepiece of a surgical dissecting microscope (Zeiss Op-Mi6, Oberkochen, West Germany) displayed on a 25-inch video monitor (NEC, Melville, NY). The wound area was recorded for approximately 30 s with a calibration ruler in the field of view using a videocassette recorder (JVC HR J44SU, Elmwood Park, NJ). The wound was videotaped on Day...
0 and then on alternate days from Days 2 through 16. The animal remained in this prone position for approximately 30 s, until the wound had been appropriately recorded. The wounds were dressed again and the experimental agents administered.

The videotaped images were digitized into a 200 MHz personal computer (Gateway, North Sioux, City, SD) at a resolution of 1500 × 1125 pixels using a Snappy Video Snapshot-capturing device (Play, Inc., Rancho Cordova, CA). The digitized images were imported into a data analysis program (OPTIMAS 4.0, Bioscan Inc., Edmonds, WA) where wound area measurements were made by tracing the wound margins with a fine-resolution computer mouse. The wound margins to be traced were determined by comparing the chromatic differential of the wound with the surrounding tissue. The area that was traced then provided a calculation of the area within the traced boundaries. The same investigator performed all wound area tracings.

**Histology**

Histologic examination of excised wounds was performed by light microscopy. Thin sections of experimental and control wounds were stained with hematoxylin and eosin.

**Experimental Groups and Statistical Analysis**

Three experimental designs were used to determine the effects that TNP-470 has upon the rate of wound closure. First, we compared the effects of three concentrations of TNP-470 (5.0, 0.5, and 0.05 mg/kg) and the vehicle control were administered on Days 0, 2, and 4. Significant differences were observed for all concentrations versus the control and among the various concentrations of TNP-470 (P < 0.05). Error bars are based on standard error measurements.

**TNP-470 alone.** Three concentrations of TNP-470 were formulated: 5.0, 0.5, and 0.05 mg/kg. These concentrations were selected from earlier studies on dosing and were well below levels at which toxic side effects have been described (personal communication with TAP Holdings Inc., Deerfield, IL). Either TNP-470 dissolved in 2% ethanol and diluted in 0.9% normal saline or a vehicle control solution of 2% ethanol diluted in 0.9% normal saline was injected subcutaneously into the femoral triangle. The injections were made at a volume of 0.1 cc using a 30-gauge needle. Three concentrations of TNP-470 (5.0, 0.5, and 0.05 mg/kg) and the vehicle control were injected on Days 0, 2, and 4. Also, in separate animals the three concentrations (5.0, 0.5, and 0.05 mg/kg) along with the vehicle control were administered daily on Days 0 through 6. The three concentrations of TNP-470 were analyzed versus each other and versus a control injection of a 2% ethanol solution. The data from this group were analyzed by a repeated measures ANOVA followed by Tukey's post hoc analysis and Student's t test. In addition, H&E staining was performed on wounds excised from the 5.0 mg/kg concentration group and compared to excised wounds from the vehicle control group for the 0, 2, 4 dosing regimen.

**TNP-470 + minocycline.** TNP-470 and minocycline were coadministered. Two concentrations and routes of administration were performed. First, a 5.0 mg/kg concentration of TNP-470 was injected subcutaneously on Days 0, 2, and 4 and a 10.0 mg/kg concentration of minocycline diluted in 0.9% normal saline was administered into the intraperitoneal space on Days 0, 1, 2. The control groups received 5.0 mg/kg TNP-470 administered with a 0.9% normal saline intraperitoneal injection. Second, a 5.0 mg/kg concentration of TNP-470 was injected subcutaneously on Days 0, 2, and 4 and a 4.0 mg/kg concentration of minocycline diluted in 0.9% normal saline was administered via an orogastric feeding tube on Days 0, 1, and 2. The control groups received 5.0 mg/kg TNP-470 administered with a 0.9% normal saline solution administered via an orogastric feeding tube. These groups were analyzed by Student’s t test.
TNP-470 + exogenous bFGF. The combined administration of TNP-470 and exogenous bFGF (R&D Systems, Minneapolis, MN) was performed. A 5.0 mg/kg concentration of TNP-470 was administered subcutaneously on Days 0, 2, and 4. Exogenous bFGF was administered topically at 1.0 μg/wound in 0.25 g DuoDerm on Days 0, 1, and 2. The control groups consisted of 5.0 mg/kg of TNP-470 + the DuoDerm vehicle, 1.0 μg/wound of exogenous bFGF coadministered with an injection of a 2% ethanol solution, and the administration of both vehicles. The data were analyzed by Student’s t test.

RESULTS

No significant differences were measured between the weights of experimental and control groups at any point of the study for either dosing regimen (Student’s t test). All animal groups lost weight initially; however, all groups had regained the lost weight by Day 16. No differences were noted between the experimental and control groups of either dosing regimen regarding the respiratory rate, heart rate, or tolerance of anesthesia.

TNP-470 alone. Analysis of the data from the first dosing regimen (Days 0, 2, and 4) revealed significant differences in wound areas, dependent upon the concentration of TNP-470 administered. There were significant decreases in the rate of wound closure for all concentration groups compared to the control groups (P < 0.05) (Fig. 1). Additionally, significant differences were observed among the concentration groups. Specifically, the 5.0 mg/kg concentration group had wound areas that were larger than the 0.5 mg/kg concentration group on Days 14 through 16, and larger than the 0.05 mg/kg concentration group on Days 2 through 16 (P < 0.05). Furthermore, the 0.5 mg/kg concentration group animals had wound areas that were significantly larger than the 0.05 mg/kg concentration group on Days 2 through 4 (P < 0.05). The highest concentration of TNP-470 (5.0 mg/kg) resulted in the greatest difference in average wound area, yielding wounds that were 20.4% larger than the controls over the duration of the study. The 0.5 mg/kg and 0.05 mg/kg concentration groups yielded average wound areas that exceeded the controls, by 13 and 9.1%, respectively, over the duration of the study. Histologic examination of the 5.0 mg/kg concentration group versus the vehicle control using H&E staining revealed a markedly delayed rate of wound healing in the animals treated with TNP-470 (Figs. 2 and 3). Specifically, this delay in wound heal-
FIG. 3. Delayed wound healing—Day 12 (5.0 mg/kg concentration of TNP-470). Note lack of epithelial layer over surface. Exposed surface consists of necrotic tissue overlying thin layer of granulation tissue (10× magnification).

ing was evidenced by a decreased number of granulation tissue blood vessels, increased thickness of the necrotic layer, and failure to completely reepithelialize the surface of the wound.

Analysis of the data from the second dosing regimen (Days 0 through 6) revealed that all three concentration groups again yielded significantly larger wounds when compared with the 2% ethanol control group (P < 0.05). However, additional analysis comparing the effects of administering TNP-470 on Days 0, 2, and 4 versus Days 0 through 6 showed no significant difference between the two dosing regimens at the 5.0 and 0.5 mg/kg concentrations (Student’s t test). Only the 0.05 mg/kg concentration group, when administered on Days 0 through 6, yielded significantly larger wounds on Days 2 through 4 versus the same concentration when administered on Days 0, 2, and 4 (P < 0.05).

TNP-470 + minocycline. Analysis of combined administration of 5.0 mg/kg concentration of TNP-470 + minocycline revealed no significant differences versus the vehicle control groups. Neither administration via intraperitoneal injection (average P value 0.36) nor orogastric delivery (average P value 0.41) yielded wound areas that were significantly different versus controls.

TNP-470 + exogenous bFGF. Topical bFGF alone resulted in significantly decreased wound areas compared to controls (P < 0.05) (Fig. 4). The combined administration of 5.0 mg/kg concentration of TNP-470 and exogenous bFGF yielded wound areas that are significantly smaller (P < 0.05) than TNP-470 alone but significantly larger (P < 0.05) than bFGF alone. Additionally, the combined administration yielded no significant differences versus the vehicle control group except on Day 2.

In the experiments that measured the effect of TNP-470 alone and the effect of TNP-470 + minocycline, the experimental and control animals were dressed with petrolatum gauze and Tegaderm. In contrast, the animals in the TNP-470 + exogenous bFGF experiment were dressed with Duoderm gel and Tegaderm. The reason for this change in primary dressing was that we were concerned about the accuracy of topical administration of exogenous bFGF in the presence of petrolatum gauze. Specifically, we were concerned that some of the exogenous bFGF would be absorbed into the
gauze with a subsequent lowering of the bFGF concentration at the wound site. Statistical analysis has demonstrated that there is no significant difference in the effect of a 5.0 mg/kg concentration of TNP-470 when administered with petrolatum gauze or Duoderm gel as the primary dressing. Also, each experiment that was conducted had an appropriate control group for statistical comparison against the experimental groups. Both primary dressings (petrolatum gauze and Duoderm gel) serve to provide a moist wound healing environment that has been demonstrated to facilitate wound healing and ease dressing changes.

**DISCUSSION**

Our results show that TNP-470 is capable of exhibiting a dose-dependent inhibition of murine cutaneous wound healing by secondary intention. In our study, the greatest effect was obtained by administration of a 5.0 mg/kg dose, which yielded wound areas that were more than 20% larger than control wounds. Our largest dose was below the maximum tolerated dose of 6 mg/kg and the lethal dose of 256 mg/kg [personal communication with TAP Holdings Inc., Deerfield, IL]. We viewed no significant difference in the physiologic parameters between experimental and control groups, suggesting that TNP-470 was capable of modulating wound healing via distant subcutaneous delivery with no noticeable systemic toxicity. We believe this has significant implications in several areas. First, TNP-470 is currently undergoing clinical trials in the treatment of several diseases. It could be anticipated that many patients potentially could benefit from the use of this agent to control their disease. These patients would undoubtedly be subject to wound healing problems from a variety of sources, including surgical intervention to further eradicate disease, that could be potentially affected by treatment with TNP-470. Secondly, there are situations in which excessive wound healing leads to an unappealing aesthetic results or functional disability. TNP-470 may be of benefit in those special situations.

We made two attempts to increase TNP-470’s effect on wound healing without success. Daily therapy showed little advantage after alternate-day therapy within the first 6 days. This is not surprising when one considers that the half-life of TNP-470’s primary metabolite is 48 h [personal communication with TAP Holdings Inc., Deerfield, IL]. Further, an attempt to augment the wound closure inhibition of TNP-470 by coadministration with intraperitoneal and oral minocycline was unsuccessful. A possible reason for minocycline’s lack of success may be that its ability to augment TNP-470 is limited to its known effect on neoplastic disease.

The final phase of our experiment showed that coadministration of TNP-470 given systemically along with exogenous bFGF given locally yielded rates of wound healing that were not significantly different versus the vehicle controls. Error bars are based on standard error measurements.
closure that were not significantly different when compared with those of controls. This implies that bFGF can reverse TNP-470-induced inhibition of wound healing. This benefit would certainly be useful in the clinical setting where bFGF could be administered topically to cutaneous wounds to normalize the rate of wound closure in a patient who is receiving TNP-470 as a therapeutic agent to treat a neoplastic disease. Basic FGF is considered the prototypical angiogenic agent, in which direct induction of capillary sprouts in vivo and in vitro models of angiogenesis has been demonstrated [16, 17]. Interactions between TNP-470 and bFGF have been previously described [8, 11]. TNP-470 has been shown to potentially inhibit endothelial cell proliferation induced by both bFGF and vascular endothelial growth factor (VEGF) [18]. Also, the growth of bFGF transfected MCF-7 breast carcinoma cells is inhibited by treatment with TNP-470 [19, 20]. Mechanisms underlying the angiogenic inhibitory properties of TNP-470 have been recently elucidated by two independent laboratories [21, 22]. TNP-470 has been shown to bind to a metalloprotease, methionine aminopeptidase (Met AP-2). The inhibition of Met AP-2 enzymatic activity appears to be responsible for the antiproliferative effects of TNP-470. However, the exact nature of the interaction between bFGF and TNP-470 awaits further clarification.

REFERENCES