

VEGF Trapon inhibits tumor growth in papillary thyroid carcinoma

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Abstract. – **OBJECTIVE:** Vascular endothelial growth factor (VEGF) is the most potent inducer of neovasculature, and its increased expression has been related to a worse clinical outcome in many disease. Angiogenesis from thyroid cancer cell plays the important roles in post-surgical persistent, recurrent, and metastatic papillary thyroid cancer (PTC). Vascular endothelial growth factor (VEGF) Trapon is a newly developed VEGF-blocking agent with stronger affinity and broader activity than the anti-VEGF antibody bevacizumab. In this study, we tested the activity of VEGF Trapon on a PTC model in vivo.

MATERIALS AND METHODS: BC-PAP (derived from papillary carcinomas) transfected with a luciferase-expressing vector were injected into the back to mice. I.p. treatment with VEGF Trapon or control protein (25 mg/kg twice weekly) was started shortly after tumor injection to prevent tumor development (prevention model) or after established tumors were formed to inhibit tumor growth and metastasis formation (intervention model).

RESULTS: In the prevention model, VEGF Trapon inhibited tumor growth by $73 \pm 12\%$ compared with control ($p = 0.014$) and significantly prolonged survival. In the intervention model, VEGF Trapon inhibited tumor growth by $68 \pm 7\%$ ($p < 0.01$). Microvascular density was reduced by 56% due to VEGF Trapon treatment ($p < 0.01$).

CONCLUSIONS: VEGF Trapon is a potent inhibitor of BC-PAP tumor growth, angiogenesis and blocks the biological function of VEGF in vivo. These results support further clinical development of VEGF Trapon for PTC and other cancer types.

Key Words:

Thyroid carcinoma, Vascular endothelial growth factor, Angiogenesis.

Introduction

Thyroid cancers of follicular cell origin are the most common endocrine malignancies¹. Although the survival rate of patients with well-differentiated thyroid cancer exceeds the rate for most other cancers, the development of metastasis continues to be the most significant cause in thyroid cancer mortality¹. Multiple pathogenic steps are involved in tumour invasion and metastases. These include the proliferation and detachment of tumour cells from the primary neoplasm, invasion of the surrounding extracellular matrix, angiogenesis, vascular or lymphatic dissemination, and eventually, homing of the tumour cells and proliferation at the new sites^{2,3}.

It has previously demonstrated that VEGF was highly expressed in thyroid carcinoma and was associated with high metastatic potential and advanced disease stage⁴⁻⁶. Vascular endothelial growth factor (VEGF) is a critical component of both physiologic and pathologic angiogenesis. When VEGF interacts with the VEGF-receptor expressed in endothelial cells, it leads to the proliferation, migration, and stabilization of normal blood vessels⁷. The pharmacologic inhibition of angiogenesis via the VEGF pathway is an important therapeutic target that prevents cancer growth and metastasis formation⁸.

VEGF Trapon is a soluble decoy receptor comprising parts of VEGFR-1 and VEGFR-2 based on a human IgG1 backbone⁹. After modifications of the parent compound that had several interactions with the extracellular matrix, VEGF TraponR1R2 (VEGF Trapon) has been developed. It avidly binds VEGF and leads to potent suppression of VEGF signaling and angiogenesis

at low concentrations (picomolar range). This agent is currently under clinical investigation. Enhanced clinical activity of VEGF Trapon compared with the monoclonal antibody bevacizumab against VEGF is expected because VEGF Trapon recognizes the whole VEGF family that binds to VEGFR-1 and VEGFR-2, including placental growth factor, and it possesses higher affinity for VEGF than bevacizumab *in vitro*. In preclinical models, VEGF Trapon has been shown to potently inhibit tumor growth, metastasis formation, and ascites formation in several murine tumor models^{10,13}.

In the present study, we tested the activity of VEGF Trapon on tumor growth and spontaneous metastasis formation in a PTC model *in vivo*. We found that VEGF Trapon is a potent inhibitor of primary tumor growth and metastasis formation and significantly prolongs survival of tumor-bearing mice.

Materials and Methods

Cell line and reagents

BC-PAP cells were obtained from the American Type Culture Collection. The cells were cultured in DMEM containing 10% fetal bovine serum, 2 mM⁻¹ glutamine (Life Technologies), 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C in humidified atmosphere containing 5% CO₂. VEGF Trapon and its control IgG backbone (hFc) were kindly provided by Regeneron Tarrytown, NY, USA.

BC-PAP Proliferation *in vitro*

Cell growth inhibition by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. BC-PAP cells were seeded at a density of 3 × 10³ cells per well in 96-well microtiter culture plates. After overnight incubation, medium was removed and replaced with fresh medium containing with VEGF Trapon and control (1 µg/mL). On completion of 72 hours of incubation, 20 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/mL in PBS) were added to each well and incubated further for 2 hours. Upon termination, the supernatant was aspirated and the MTT formazan formed by metabolically viable cells was dissolved in 100 µL of isopropanol. The plates were mixed for 30 minutes on a gyratory shaker, and absorbance was measured at 595 nm using a plate reader (TECAN, Durham, NC, USA).

Terminal dUTP Nick-end Labeling (TUNEL) Assay

Cells were cultured on chamber slides and allowed to adhere overnight, and they were then treated with VEGF Trapon and control at concentrations of 1 µg/mL for 24 h. Apoptosis of the cells was evaluated on the basis of the TUNEL assay using the Dead End Fluorometric TUNEL System (Promega, Madison, WI, USA) according to the manufacturer's instructions. All assays were performed in quadruplicate.

Subcutaneous Xenografted BC-PAP Tumor Model

Luciferase-transfected BC-PAP cells (5 × 10⁵) mixed with Matrigel were injected into the back of the BALB/c female mice. On a weekly basis, tumor imaging was done by i.p. administration of luciferin and by using bioluminescence technology (Xenogen system). Before starting the treatment, mice were grouped according to tumor burden as determined by luciferase expression. As an indicator of treatment-related toxicity, mouse weights were measured once weekly. Primary tumor weights were determined at the end of the study when mice were sacrificed. All experiments were repeated at least twice. Representative experiments are shown.

Prevention Experiments

Treatment with 10 mg/kg VEGF Trapon or its control by i.p. injections twice weekly was started on day 3 after implantation of the tumor and mice were treated for 30 days. In these experiments, survival of mice was determined as well.

Intervention Experiments

Treatment was started when tumor burden had been clearly increased according to luciferase expression measurements. Treatment with 25 mg/kg VEGF Trapon or its vehicle control started on day 14 after tumor implantation and mice were treated for 22 days. In the model, VEGF Trapon and control IgG were given in a blinded fashion and the code was broken by Regeneron after completion of the experiments.

Immunohistochemistry

Immunohistochemical studies were done on 4-µm-thick sections derived from zinc-fixed, paraffin wax-embedded tumor tissue blocks. These tumors were harvested at the end of the experiments (after 22 days). Sections were subsequently dewaxed, rehydrated, and had endogenous per-

oxidase activity quenched before specific immunohistochemical staining. After specific staining or H&E staining, sections were dehydrated in alcohol and xylene and subsequently mounted. TUNEL staining for tumor tissue was based on the protocol of the Dead End Colorimetric TUNEL System (Promega). The tissue sections were viewed at x100 magnification and images were captured with a digital camera. Four fields per section were analyzed, excluding peripheral connective tissue and necrotic regions. Microvessel density (MVD) in each field was defined as the mean number of microvessels containing high levels of CD31-stained microvessels. Percentage of apoptotic cells was defined as TUNEL-positive cells among 1000 tumor cells. Mean values of MVD and percentage of TUNEL-positive cells in each group were calculated from three tumor specimens.

Statistical Analysis

Results were analyzed with the Excel software using Student's *t* test, with $p < 0.05$ considered as statistical significant.

Results

VEGF Trapon Inhibits Proliferation of BC-PAP Cells

Treatment of BC-PAP cells with VEGF Trapon for 72 hrs resulted in potent growth inhibition in a dose-dependent manner for the BC-PAP lines studied (Figure 1A).

VEGF Trapon Induces Apoptosis in BC-PAP Cells.

BC-PAP cells were treated with VEGF Trapon (1 $\mu\text{g}/\text{mL}$) for 72 hrs, the TUNEL assay showed that the percentages of TUNEL-positive cells were 1.2% without treatment and 13% with 1 $\mu\text{g}/\text{mL}$ VEGF Trapon treatment (Figures 1B).

In vivo Antitumor Activity of VEGF Trapon Against s.c. Xenografted BC-PAP Tumors

To determine the effect VEGF Trapon on tumor development *in vivo*, treatment of mice was started after 4 days following tumor implantation (prevention model). Tumor growth was inhibited by $73 \pm 12\%$ ($n = 10$; $p = 0.014$) as measured by luciferase expression (Figure 2A). Survival in these animals was also monitored. VEGF Trapon treatment significantly prolonged survival of

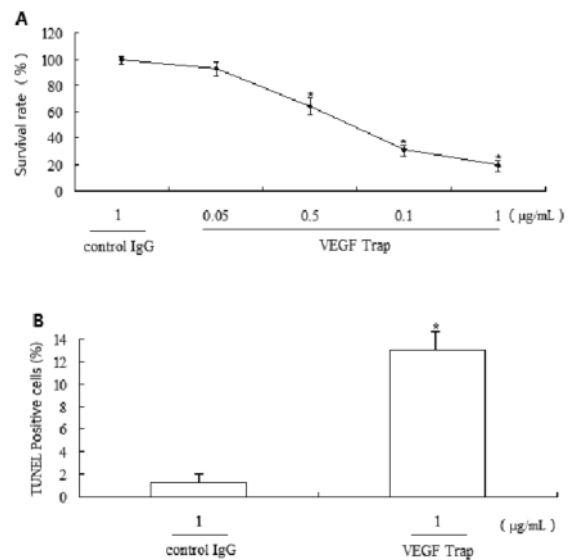


Figure 1. Effect of VEGF Trapon on proliferation and apoptosis in BC-PAP cells. **A**, BC-PAP cells were either untreated or treated with increasing concentration of VEGF Trapon for 72 hs and analyzed for viable cells by MTT assay as described in Materials and Methods. **B**, BC-PAP cells were treated with 1 $\mu\text{g}/\text{mL}$ VEGF Trapon or 1 $\mu\text{g}/\text{mL}$ control IgG for 72 hs and analyzed for apoptotic cells by TUNEL assay as described in Materials and Methods. Vs control, * $p < 0.05$.

these animals after 29 days of treatment (twice weekly) by 27 days compared with controls. Survival of VEGF Trapon was 56 ± 8 days versus 32 ± 3 days in controls ($n=10$ in each group; $p < 0.001$; Figure 2B). No significant changes in weight of the treated animals were observed during treatment.

In the intervention model, treatment with VEGF Trapon doses of 25 mg/kg twice weekly *i.p.* inhibited primary tumor growth by $68 \pm 7\%$ ($p < 0.01$) as measured by luciferase expression. This inhibitory effect was confirmed by tumor weight assessment (Figure 3A-C). Formation of spontaneous lung metastases was almost completely prevented by VEGF Trapon. No significant weight changes of the treated animals were observed in these mice as well.

VEGF Trapon-induced BC-PAP Tumor Growth Inhibition is Associated with Decreased MVD

To determine whether VEGF blockade has an effect on BC-PAP tumor vasculature, MVD was analyzed. In the *s.c.* xenografted tumors of mice treated with VEGF Trapon, the expression levels

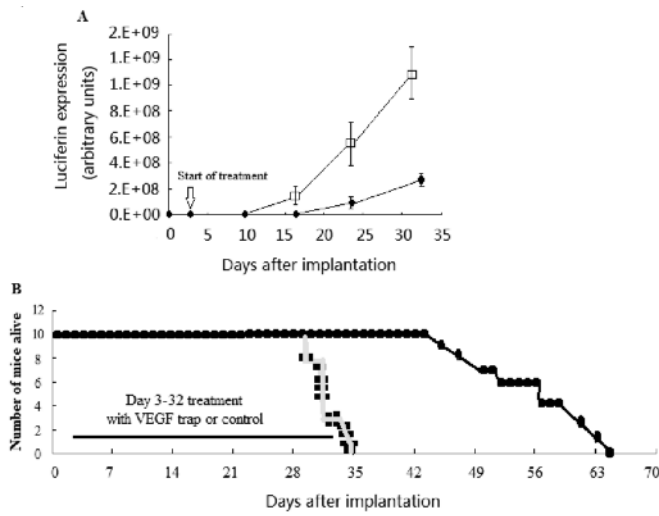


Figure 2. Delay of tumor development and prolonged survival by treatment with VEGF Trapon. **A**, after implantation of BC-PAP cells in the back of mice, VEGF Trapon (black dots) and control treatment (black squares) were given in a blinded fashion starting day 4. Tumor growth was inhibited by $73 \pm 12\%$ ($n = 10$; $p = 0.014$) as measured by luciferase expression 32 d after tumor implantation. **B**, survival in these animals was monitored. Survival of VEGF Trapon-treated mice (black dots) was 56 ± 8 d versus 32 ± 3 d in controls (black squares; $n = 10$ in each group; $p < 0.001$) after 29 d of treatment.

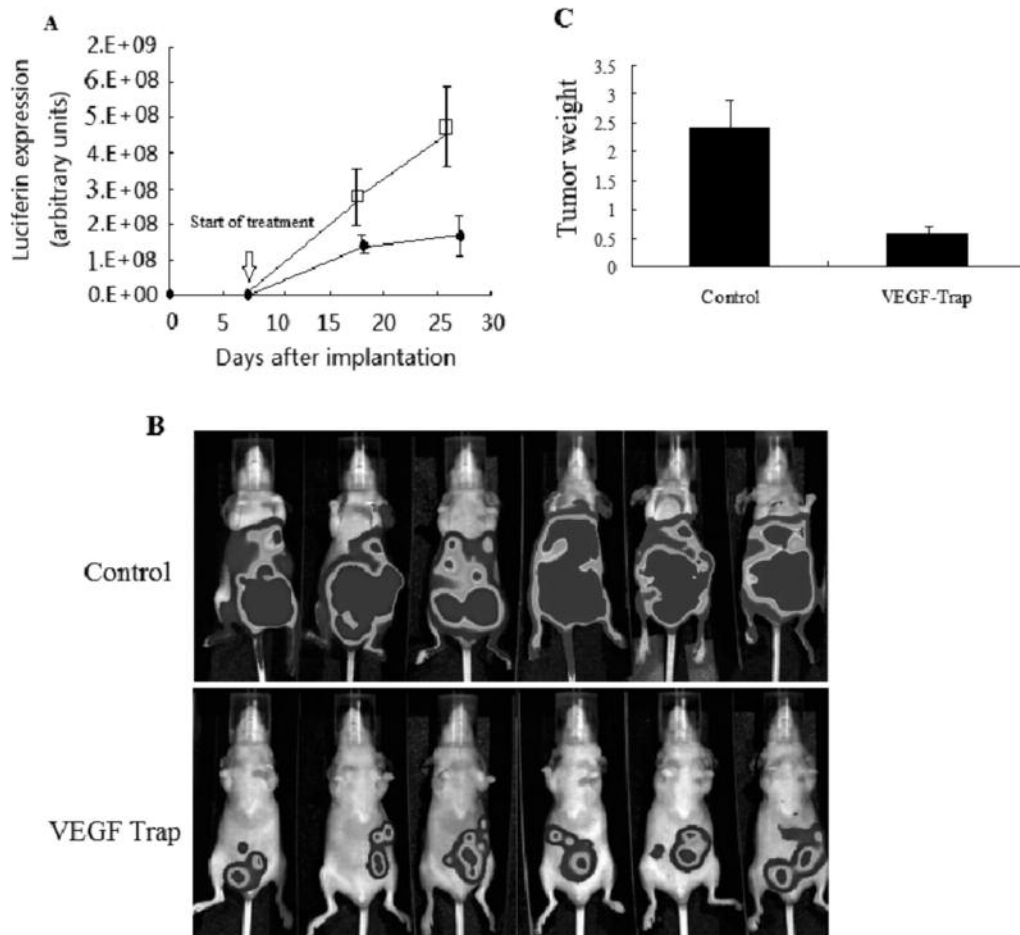


Figure 3. Tumor growth inhibition and prevention of spontaneous metastasis formation. **A**, treatment with VEGF Trapon at 25 mg/kg twice weekly i.p. (black dots) inhibited primary tumor growth by $68 \pm 7\%$ compared with controls (squares) as measured by luciferase expression. **B**, representative picture showing the difference in luciferin expression between control and VEGF Trapon-treated mice (6 mice of each group of a representative experiment). **C**, the difference in luciferin expression was confirmed (in each experiment) by tumor weight.

of MVD was significantly decreased in tumor specimens from VEGF Trapon treated groups compared to that in specimens from the vehicle-treated group, as assessed by CD31 staining. New blood vessel formation was significantly reduced in BC-PAP tumor by VEGF Trapon in the intervention model. It also shows that VEGF Trapon induced tumor cell apoptosis as measured by TUNEL staining.

Discussion

PTC are usually treated with surgical removal of the gland and, if necessary, radioablative treatment to eradicate possible neoplastic remnants¹⁴. However, 10% of patients affected by thyroid cancer will present with completely undifferentiated phenotypes or will display relapsed cancerous lesions that have acquired poorly differentiated pathologic characteristics^{15,16}. These tumors have lost both the morphologic and functional characteristics of a differentiated thyroid gland and fail to uptake and retain radioactive iodine. For these individuals, treatment with different combinations of chemotherapeutic agents presently represents the most effective therapeutic approach. Unfortunately, the majority of these tumors will present or eventually develop resistance to chemotherapy and progress to a negative outcome. Therefore, the novel therapeutic option for management of this disease is needed.

Blockade of the VEGF signaling pathway predominantly inhibits endothelial cell proliferation, migration, and survival. Initially, Senger et al¹⁷ discovered VEGF as a permeability factor with a potency of 400-fold greater than histamine and found that tumor-derived VEGF induces vascular leakage, causing extravasation of fibrinogen. The mechanism of VEGF induced vascular leakage has not been fully elucidated. VEGF has been shown to cause endothelial cell fenestrations, perturbed endothelial cell-cell interactions, and breakdown of the basal membrane, which all may increase vascular leakage¹⁸. Furthermore, altered capillary-venule hierarchy,

vascular tortuosity, and variability in vessel diameter have been described as a consequence of VEGF stimulation¹⁹. Inhibition of the VEGF signaling cascade can partially normalize these VEGF-induced features of tumor vessels and can reduce vessel diameter to such an extent that eventually blood flow may stop²⁰. Inhibition of VEGF by VEGF Trapon has been shown to block

endothelial cell fenestrations within 24 h¹⁹. In our study, it revealed that VEGF Trapon not only reduced MVD but also led the prevention model growth. In the model, VEGF Trapon inhibited tumor growth by 73% and 68%, respectively. Microvascular density was reduced by 56% due to VEGF Trapon treatment. Furthermore, formation of spontaneous lung metastases was almost completely prevented by VEGF Trapon (98% inhibition).

An increase in the apoptotic rate of tumor and endothelial cells was expected based on previous studies²¹. In our study, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling staining did show an induction of apoptosis in BC-PAP tumor cells by VEGF Trapon treatment.

Conclusions

The results of this study indicate that VEGF Trapon is a very potent inhibitor of tumor growth and metastasis formation in a PTC model without causing major drug-related toxicity. The stainings of MVD confirmed that this agent blocks VEGF activity *in vivo*. It will be of particular interest to study the efficacy of this agent in the clinical setting of PTC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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