Systemic Administration of Vascular Endothelial Growth Factor Monoclonal Antibody Reduces the Growth of Papillary Thyroid Carcinoma in a Nude Mouse Model

Andrew J. Bauer,1 Aneeta Patel,1 Richard Terrell,1 Krishna Doniparthi,1 Motoyasu Saji,2 Matthew Ringel,2 R. Michael Tuttle,3 and Gary L. Francis1

1 Department of Pediatrics, Uniformed Services University of the Health Sciences, Bethesda, MD
2 Department of Endocrinology, Washington Hospital Center and Medstar Research Institute, Washington, DC
3 Department of Endocrinology, Memorial Sloan Kettering Cancer Center, New York, NY

Abstract. Papillary thyroid carcinomas (PTC) are the most common thyroid cancers in children. Most are successfully treated with surgery and radioactive iodine, but some persist. PTC express high levels of vascular endothelial growth factor (VEGF) and VEGF receptor (Flt-1). PTC with the most intense expression of VEGF have the greatest recurrence risk. We hypothesized that blockade of VEGF would inhibit PTC growth. To test this, we used systemic VEGF monoclonal antibody (VEGF-MAb) to treat PTC xenografts in nude mice. Treated animals (n = 9) received 200 µg VEGF-MAb by daily iv injection for 10 wk, while control animals (n = 9) received vehicle alone. Tumor size was significantly reduced in the treatment group (0.28 ± 0.06 vs 1.05 ± 0.25 g, p = 0.008). VEGF immunostaining was more intense (2.57 ± 0.30 vs 1.75 ± 0.25, p = 0.06) and the number of p53 positive cells was increased (1.66 ± 0.24 vs 0.83 ± 0.31, p = 0.048) in treated tumors. Animal weight was similar in both groups (29.1 ± 1.1 vs 27.4 ± 1.1 g, p = 0.30). In conclusion, systemic VEGF-MAb significantly reduced the growth of PTC, suggesting that VEGF-MAb might be useful for treatment of resistant PTC. (received 14 February 2003, accepted 27 March 2003)

Keywords: thyroid cancer, papillary thyroid carcinoma, vascular endothelial growth factor

Introduction

Papillary thyroid carcinomas (PTC) are the most common thyroid cancers of children and adolescents [1-6]. In most series, they account for 60 - 80% of cases [1-7]. Conventional treatment, including total thyroidectomy and radioactive iodine ablation, results in favorable overall survival. Many series report disease-specific mortality <10% and in some series mortality of 1% has been seen [5,7-9]. Despite the overall success of this approach, some patients have persistent disease despite treatment with the maximal recommended life-time doses of radioactive iodine [5,10]. Other patients have detectable serum thyroglobulin (Tg), indicating persistent disease, but no disease that can be imaged with radioactive iodine. Treatment for these patients is highly controversial [11]. For these reasons, novel treatments are needed to assist in the management of children with persistent PTC.

Seminal work by Folkman and others [12-21], showed that growth of solid tumors is dependent on formation of new blood vessels, ie, angiogenesis. According to this theory, tumors secrete angiogenic factors that induce proliferation of new vessels, sustaining tumor growth. In many studies, vascular endothelial growth factor (VEGF) has been identified as one of the most important angiogenic stimuli [22-24]. We have shown that VEGF is important for the growth and recurrence of PTC in...
children and adults [25]. PTC with the most intense expression of VEGF were the largest, and had the highest risk of recurrence [25]. Based on the importance of VEGF, we hypothesized that VEGF blockade might reduce the growth of PTC. We tested this by using VEGF monoclonal antibody (VEGF-MAb) to treat PTC xenografts in nude mice. Our data show that VEGF-MAb significantly reduces the growth of PTC xenografts.

Materials and Methods

Materials. Human vascular endothelial growth factor monoclonal antibody (VEGF-MAb, #33102-95, Genentech, Inc., South San Francisco, CA) was administered by daily ip injection at a dose of 200 µg/day [26-28].

Cell line and cell viability and proliferation. NPA cells (a generous gift from Dr. G. Juillard, University of California, Los Angeles, CA) were propagated using Dulbecco’s modified Eagle’s Media supplemented with 10% fetal bovine serum, sodium bicarbonate (0.375%), glutamine (2 mM), non-essential amino acids (1 X), and gentamycin (0.01 mg/ml) in an atmosphere of 5% CO2/air (95% humidity, 37°C). Direct effects of VEGF-MAb on the growth and viability of NPA cells were examined in vitro. Replicate cultures (n = 12 for each group) containing 1x10^6 cells were incubated in serum-free media (control, SFM) or SFM containing VEGF-MAb (60 µg/ml) for 24 hr. Following incubation, cell number and viability were determined by trypan-blue exclusion.

Animal procedures. Following approval by the Institutional Animal Care and Use Committee, Department of Laboratory and Animal Medicine, Uniformed Services University of the Health Sciences, Bethesda, MD, 18 BALB/c nu/nu mice were anesthetized (7.3 mg ketamine hydrochloride and 2.3 mg xylazine /100 gm body wt, ip) and placed over a warming pad. Using aseptic technique, mice were injected subcutaneously with 1 x 10^6 NPA cells over the dorsal scapula. Cells were allowed to implant for 7 days. On day 7, mice were randomized into 2 groups (n = 9 for each treatment) to receive one of the following treatments by daily ip injection for 10 wk: (a) Negative Control Group, vehicle only (0.3 ml of 0.01% Tween 80 in PBS); (b) VEGF-MAb (0.3 ml in PBS, 200 µg daily) [26-28].

Tumor size was determined weekly by skin caliper measurements, and calculated using the formula for the volume of an ellipse (mm^3) = (length x height x width)/2. Animals were allowed ad libitum access to food and water and were weighed weekly. After 10 wk, the animals were sacrificed. Tumors were removed, weighed, and divided. Portions were fixed in formalin for routine histology and immunohistochemistry.

Immunohistochemical staining. Sections (5 µm) were prepared from each tissue block. The first was stained for routine histology (hematoxylin/eosin) to assess the extent of necrosis. The ratio of viable cells:total cells was used to determine the percent necrosis. Adjacent sections were stained for VEGF (Cat #A20; Santa Cruz Biotechnology, Santa Cruz, CA) and p53. The p53 antibody was specific for epitopes common to mutant and wild type p53 (cat #250-2542 clone BP53-11, Ventana, Tucson, AZ).

For both VEGF and p53, sections were rehydrated through a series of graded alcohols and water. Antigen retrieval was performed in citrate buffer (pH 6.0, 100°C, 30 min) and the sections were incubated in universal tissue blocker to inhibit non-specific binding. Endogenous peroxidase was quenched (3% hydrogen peroxide, 30 min) and the sections were incubated with specific primary antibodies (1:50 dilution for VEGF; p53 was prediluted by the manufacturer), followed sequentially by secondary biotinylated antibody, horseradish peroxidase, and dianinobenzidine chromogen (Ventana Nexes Automated Immunostainer, Tucson, AZ).

Staining was quantified by 2 independent investigators, who were blinded to the animal number and treatment. VEGF staining was graded as follows: grade 0 = absent, grade 1 = minimal, grade 2 = moderate, and grade 3 = intense. p53 staining was graded as grade 0 = absent, grade 1 = <10 positive cells/high powered field (HPF), grade 2 = 11-20 positive cells/HPF and grade 3 = >20 positive cells/HPF. Colorectal carcinoma was used as a positive control for VEGF and p53 staining. Negative
Fig. 1. The effect of VEGF-MAb on tumor volume. Animals (n = 9/group) received vehicle (Control) or VEGF-MAb (200 µg) by daily ip injection. Tumor volume was determined weekly as (length x width x height)/2. VEGF-MAb reduced tumor growth from wk 4 through wk 10 (p = 0.011 at 10 wk).

Fig. 2. The effect of VEGF-MAb on tumor weight. Animals (n = 9/group) received vehicle (Control) or VEGF-MAb (200 µg) by daily ip injection. Animals were sacrificed after 10 wk of treatment; the tumors were removed and weighed. VEGF-MAb significantly reduced tumor weight (p = 0.008).

Fig. 3. Relationship between animal weight and well-being. Representative animals are shown at 7 wk of treatment. Both control and treated animals appeared well, remained active, and groomed appropriately throughout the 10 wk study.
Fig. 4. The effect of VEGF-MAb on the extent of necrosis. Animals (n = 9/group) received vehicle (Control) or VEGF-MAb (200 µg) by daily ip injection. The mean % necrosis for the control tumors was greater than in the VEGF-MAb treated group (p = 0.022).

Fig. 5. The effect of VEGF-MAb on tumor histology, VEGF and p53 staining. Staining for routine histology (A and D), VEGF (B and E), and p53 (C and F) are shown for control (A, B, and C) and VEGF-MAb (D, E, and F) treated animals. Compared to control, the VEGF-MAb treated tumors displayed smaller areas of necrosis (A and D) but more diffuse and intense VEGF staining (B and E), as well as more numerous p53 positive cells (C and F). (All at 100 x original magnification.)

Fig. 6. The effect of VEGF-MAb on VEGF expression. VEGF immunostaining was more intense in VEGF-MAb treated tumors (2.57 ± 0.30, relative intensity units) compared to controls (1.75 ± 0.25, p = 0.065). In all tumors, the immediate subcapsular area showed a margin of viable cells, devoid of VEGF staining.
controls included pre-adsorption of the primary antibodies with specific blocking peptides, as well as sequential elimination of the primary and secondary antibodies.

**Data analysis.** Tumor size, animal weight, intensity of VEGF staining and staining grade of p53 expression were compared between treatments using ANOVA. Tumor growth over time was compared between treatments by repeated measures ANOVA. In addition, intensity of VEGF staining and grade of p53 expression were correlated with tumor size (linear-by-linear analysis) and the extent of necrosis (Pearson correlation).

Statistical analyses were performed using SPSS for Windows 95 (version 7.5, SPSS Inc., Chicago, IL). Non-parametric analyses were performed using the Chi-square or Fisher’s exact test as indicated. Unless otherwise stated, data are presented as mean ± SE, with 95% confidence intervals where appropriate.

**Results**

**Effects of VEGF-MAb on tumor growth.** Beginning at 4 wk, tumor volume in the VEGF-MAb treated animals (477 ± 85 mm³) was reduced 68% compared to controls (1,500 ± 346 mm³, p = 0.011). After sacrifice, tumor weight was determined. The tumor weight was significantly reduced in the VEGF-MAb treated group (0.28 ± 0.06 g) compared to controls (1.05 ± 0.25 g, p = 0.008; Fig. 2).

**Effects of VEGF-MAb on animal weight.** The initial mean weight of the control animals (27.2 ± 0.84 g) was similar to that of the VEGF-MAb treated group (25.9 ± 0.79 g, p = 0.26). During the 10 wk of treatment, animal weights remained similar in the control (27.4 ± 1.1 g) and treated animals (29.1 ± 1.1 g, p = 0.3). In addition, both groups of animals exhibited appropriate grooming and activity levels and appeared generally well (Fig. 3).

**Effects of VEGF-MAb on extent of necrosis.** The mean percent necrosis in the control tumors (63 ± 6.3%) was significantly greater than that in the VEGF-MAb treated group (30.0 ± 8.6%, p = 0.022; Fig. 4). Of interest, the immediate subcapsular areas of the control and treated tumors were devoid of necrosis (Fig. 5).

**VEGF expression.** VEGF immunostaining was more intense in tumors treated with VEGF-MAb (2.57 ± 0.30, relative intensity units; p = 0.065), compared to controls (1.75 ± 0.25) (Fig. 6). However, the difference only approached statistical significance. In the control tumors, minimal VEGF staining was detected in a patchy distribution (Fig. 5). In contrast, VEGF-MAb treated tumors showed diffuse, intense VEGF staining throughout the entire tumor (Fig. 5). In all tumors, the immediate subcapsular area showed a margin of viable cells devoid of VEGF staining that was similar in location to the regions that did not evidence necrosis. There was a tendency for tumors with more extensive necrosis to display more intense VEGF staining (r = 0.42, p = 0.19, linear-by-linear association).

**p53 expression.** Nuclear staining for p53 was detected in a similar proportion of the control (4/6, 67%; 95% CI = 0.24–0.94) and VEGF-MAb treated tumors (5/9 or 56%; 95% CI = 0.23–0.85). The
average grade of p53 staining was significantly greater in the VEGF-MAb (1.67 ± 0.24) compared to control (0.83 ± 0.31, p = 0.048) groups (Fig. 7), consistent either with increased expression of immunoreactive p53 or increased stability of total p53 protein.

**In vitro effects of VEGF-MAb.** Examination of the in vitro effects of VEGF-MAb on NPA cell cultures revealed no change in either cell number or viability. Cell viability was >99% in both control and treated cultures. By 24 hr, the number of cells in the VEGF-MAb treated cultures (8.1 ± 4.5 x 10^6) was similar to that in the control cultures (9.6 ± 2.7 x 10^6; p = 0.61), suggesting a requirement of locally produced VEGF from non-thyroidal cancer cells for VEGF mediated growth effects.

**Discussion**

VEGF is produced by a variety of different histologic variants of thyroid cancer. PTC is the most common form of thyroid cancer in children, and previous studies from our laboratory have shown that VEGF is critical for the growth and recurrence of PTC in children [25]. Based on the importance of VEGF in thyroid cancer, 2 previous studies examined the effect of VEGF blockade on the growth of thyroid cancer xenografts. The first study by Soh et al [29] used VEGF-MAb to treat the follicular thyroid cancer cell line, FTC-133. VEGF-MAb was injected ip twice weekly for 5 wk. Tumors from the VEGF-MAb treated mice were smaller (0.09 ± 0.02 g) than control (4.0 ± 0.72 g; p < 0.001). The second study by Bauer et al [30], used VEGF-MAb to treat anaplastic thyroid cancer xenografts in a similar nude mouse model. Tumors treated with VEGF-MAb were also smaller (by 60%) than controls (p = 0.017). In addition, treated animals gained weight and displayed improved levels of activity and grooming. Immunostaining for VEGF and p53 were increased, suggesting the possibility that tumor hypoxia might be increased by VEGF-MAb treatment [31-35]. Although tumor growth has not been inhibited completely in any of these studies, the reduction in tumor volume is significant and in some cases dramatic [29]. For patients with no other available treatment, this may offer an important adjunctive therapy. Neither of the previous studies determined whether VEGF-MAb would have an effect on PTC. Based on the success of those studies, we hypothesized that VEGF-MAb might also be useful against PTC. To our knowledge this is the first study to address this question.

Our data show that systemic administration of VEGF-MAb significantly reduced tumor growth (VEGF-MAb vs control, p = 0.008). These findings are consistent with the 2 prior reports that used VEGF-MAb for treatment of follicular and anaplastic thyroid cancers [29,30] and broaden the potential scope of VEGF-MAb utility.

In addition, our data suggest that the expression of VEGF and the tumor suppressor protein, p53, are increased by VEGF-MAb therapy. These data are consistent with our previous findings in VEGF-MAb treated anaplastic thyroid cancer, and support the possibility that VEGF-MAb treatment interrupts new blood vessel formation and induces tumor hypoxia [29,36,37]. In some tumors, such as non-small cell lung cancer, prostate cancer, and breast cancer, the expression of VEGF and p53 have been linked, as both are increased by hypoxia [31-34]. In these tumors, expression of p53 and VEGF have been upregulated by the hypoxia-induced transcription factor (HIF-1α). Additional study of VEGF-MAb treated PTC will be required to determine if HIF-1α is increased, and if HIF-1α specifically regulates VEGF or p53 expression in thyroid cancers.

We were concerned about the possibility that systemic administration of VEGF-MAb might reduce proliferation of all new blood vessels, and thus reduce the growth of young animals. This was not observed in our short-term study. Animal weight gain increased at a normal rate in all treated animals, and there were no unexpected deaths. It is possible that VEGF-MAb might have more potent effects against the immature and unstable vessels typical of malignant tumors [24,38]. The more mature, stable blood vessels of typical normal tissues might be less sensitive. In support of this hypothesis, it is believed that VEGF induces proliferation of endothelial cells and promotes new blood vessel formation, but other factors such as angiopoietin-1 (ANG-1) are required to stabilize these new vessels [24]. We did not
determine the expression of ANG-1 in this study. ANG-1 levels might possibly have been lower in the thyroid cancer xenografts than in normal body tissues, allowing for increased sensitivity to VEGF-MAb.

We did not find any in vitro effects of VEGF-MAb on PTC cell cultures. Cell viability and proliferation were similar in control and treated cultures. These observations support the hypothesis that VEGF-MAb works solely to inhibit angiogenesis and thereby tumor growth. VEGF-MAb does not appear to have any direct cytotoxic effect.

The fact that more extensive necrosis was found in control tumors is perplexing. It is possible that VEGF-MAb may have slowed tumor growth to such an extent that necrosis did not occur. According to this theory, tumor cells in the smaller VEGF-MAb treated tumors would have remained within a few mm of the supporting vessels. Cell death would not have been seen, resulting in reduced tumor size accompanied by continued viability and less extensive necrosis. Several alternate explanations exist, including: (a) that the increased VEGF found in treated tumors could have stimulated sufficient vessel formation to support a smaller, but viable, tumor mass, or (b) that the larger tumors were VEGF dependent earlier on, but by 10 wk (the time that the animals were sacrificed), the larger, control tumors had outgrown their blood supply, resulting in the death of the majority of cells producing VEGF and leading to our finding of increased necrosis and decreased VEGF staining. Further study is required to explore these possibilities.

In summary, our experimental data show that systemic VEGF-MAb treatment of PTC xenografts reduces tumor growth and may increase tumor hypoxia. No adverse side effects were observed. These findings suggest the possibility that VEGF-MAb might be a useful adjunct in the treatment of selected patients with persistent PTC.

Acknowledgements and Caveats

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References


