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The Role of VEGF Pathways in Human Physiologic and Pathologic Angiogenesis

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Background. **In preclinical models, VEGF is a potent stimulant of both physiologic and pathologic angiogenesis. Conversely, anti-VEGF regimens have successfully inhibited angiogenesis both *in vitro* and *in vivo*. We hypothesized that VEGF would stimulate both physiologic and pathologic angiogenesis in a human-based fibrin-thrombin clot angiogenesis assay. We further speculated that anti-VEGF regimens would inhibit angiogenesis in this assay.**

Methods. **To test these hypotheses, discs of human placental veins (physiologic model) and fragments of human tumors (pathologic model) were embedded in fibrin-thrombin clots and treated with either VEGF-A_{165} (VEGF) or anti-VEGF pathway reagents including bevacizumab, IMC-18F1, IMC-1121, and PTK787 ($n = 30$ wells per treatment group, multiple concentrations tested in each specimen). Angiogenic responses were assessed visually using a previously validated grading scheme. The percent of tissue explants that developed angiogenic invasion into the clot (%I) as well as the extent of angiogenic growth (AI) via a semi-quantitative scale were assessed at set intervals.**

Results. **VEGF failed to stimulate angiogenesis in both the physiologic and the pathologic model. While anti-VEGF reagents that targeted only one element of the VEGF pathway failed to consistently inhibit angiogenesis, PTK787, a receptor tyrosine kinase inhibitor that targets multiple VEGF and non-VEGF receptors, profoundly inhibited both physiologic and pathologic angiogenesis.**

Conclusion. **These results suggest that VEGF-related pathways may not be solely responsible for stimulating angiogenesis in humans. Targeting the VEGF pathway in combination with elements of other growth factor pathways may provide a more effective means of inhibiting angiogenesis than targeting VEGF alone.**

Key Words: **angiogenesis; human; vascular endothelial growth factor (VEGF); platelet derived growth factor (PDGF); VEGF receptors; tyrosine kinases; mechanisms.**

INTRODUCTION

The stimulus for the proliferation of new blood vessels in humans is a complex cascade of events known as the angiogenic switch [1]. This switch is turned on when the concentration of natural pro-angiogenic compounds outweighs that of antiangiogenic compounds, and it occurs in both physiologic and pathologic settings. Although numerous proangiogenic reagents have been identified as potential mediators of the angiogenic switch, the vascular endothelial growth factor (VEGF) is thought to be the most significant among them.

At least five VEGF-related ligands have been identified to date: VEGF-A, -B, -C, -D, and -E. The 165-amino acid isoform of VEGF-A is thought to be the most important in the stimulation of angiogenesis [2]. The biological effects of VEGF are conferred through its binding to three highly related receptor linked tyrosine kinases (RTKs): VEGFR-1 (Flt-1, R1), VEGFR-2 (kdr/Flk-1,

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R2), and VEGFR-3 (Flt-4, R3). The binding of VEGF to VEGFR-1 results in enhanced production of matrix metalloproteinase-9 and other tissue-specific growth factors [3]. VEGFR-2 is thought to be the primary receptor involved in angiogenesis, and its activation is considered critical in endothelial cell proliferation and survival. The VEGFR-3 receptor is involved in lymphangiogenesis and is not thought to play an essential role in endothelial cell proliferation or maturation.

Preclinical studies have repeatedly demonstrated the ability of VEGF to stimulate the proliferation and migration of endothelial cells [4–6]. Techniques used to inhibit the VEGF pathway have also been shown to be an effective means of antiangiogenesis in preclinical studies [7–9]. Studies in humans have revealed an overexpression of VEGF associated with states of human neovascularization [10]. Up-regulation of VEGF has been in healing wounds [11], human menstruation [12], and countless types of human malignancies [13]. Despite these associative findings, in humans, few data exist documenting VEGF as a direct stimulant of neovascularization in humans. Therefore, we designed a series of experiments to visually analyze the direct effect of manipulating the VEGF pathway on human angiogenesis.

We hypothesized that treatment of human tissue fragments with VEGF-A_{165} would augment angiogenic neovessel initiation and growth in human placental vein-based and human tumor-based fibrin-thrombin clot angiogenesis assays. Conversely, we speculated that treatment of these tissues with anti-VEGF reagents—those that remove VEGF from the milieu or those that block VEGFR-1 or VEGFR-2 or those that inhibit specific tyrosine kinase pathways associated with the VEGF receptors—would significantly inhibit the human angiogenic response.

METHODS

Preparation of Tissues

We used human placental veins discs as targets in a fibrin thrombin clot assay to evaluate physiologic angiogenesis. These veins possess monolayers of quiescent endothelial cells in their intima. When the veins are sliced into small discs, the mechanical trauma to the vein creates injury and a "stressful" milieu at the vessel's cut edge. Such an environment is sufficient to turn on the "angiogenic switch," stimulating these previously quiescent endothelial cells to begin to proliferate in a manner similar to that which occurs in wounding *in vivo*.

We used fragments of human tumors to evaluate pathologic angiogenesis. Unlike vein discs, which harbor resting endothelial cells, tumor fragments harbor actively proliferating endothelial cells. In addition, these fragments contain the previously formed pathologic (not physiologic) microvessels found in all invasive cancers that exceed 2 mm in diameter

All specimens obtained were freshly discarded human tissues that were not needed by the hospital for pathologic analysis. Tissues were obtained anonymously with the approval of the Institutional Review Board of Louisiana State University Health Sciences Center (LSUHSC, New Orleans, LA). Post-partum placentas and surgically harvested human tumors were obtained and transported to the lab. Placental vein tissue was processed as previously outlined [14, 15]. A series of gynecological tumors (PK) were excised in Oregon and transported by a commercial carrier to LSUHSC overnight in chilled Medium 199. Tumors were sliced into 1-mm thick segments and then 1 mm^3 fragments of fresh human tumor were fashioned using sharp dissection.

Fibrin-Thrombin Clot Angiogenesis Assay

Wells in a standard 96-well plate (Corning Inc., Corning, NY) were preloaded with a human thrombin solution (0.8 IU in 2.0 μL/well) (Sigma Chemical Company, St. Louis, MO). Architecturally intact tumor fragments or vein discs were placed into the thrombin-loaded wells. Tissue was randomly distributed among individual wells to ensure that each treatment group was representative of the entire specimen. The tissue was covered with 100 μL of a clot-forming medium containing fibrinogen (3 mg/mL) and 0.5% Σ-amino caproic acid (Sigma Chemical Company) added to nutrient medium. Nutrient medium consisted of Medium 199 and an antibiotic/antimycotic solution (100 U penicillin, 100 U streptomycin sulfate, and 0.25 μg amphotericin/mL; Gibco/Life Technologies). This mixture was incubated in a 6% CO_2, 94% air atmosphere at 37°C to allow the fibrin-thrombin clot to form. Once a tissue-containing clot was formed, 30 control wells were treated with 100 μL of a nutrient medium containing 20% fetal bovine serum (FBS) (GibcoBRL, Grand Island, NY), while experimental wells ($n = 30$ per treatment group) were treated with 100 μL of medium/FBS supplemented with test reagent. Total well volume was 200 μL. Ninety-six-well plates remained in the incubator, and nutrient or drug-containing medium was changed every seventh d.

Evaluation of Angiogenesis

Tissue-containing wells were examined under an inverted phase microscope after 14 d of incubation. Fragments were assessed to determine (1) if new blood vessels had sprouted from the fragment's cut edge and if so, (2) the degree of growth these sprouts had undertaken. This was performed using a semi-quantitative visual rating system described previously [14, 15] (Fig. 1.).

Statistics

Three measurements were of interest, each requiring different statistical analysis. The first was the proportion of wells that initiated an angiogenic response (%I). This measurement (%I) reflected the effect of the treatments on the earliest phase of angiogenesis. The angiogenic index (AI), the second measurement of interest, was treated as a continuous measurement although it is similar to a count measurement. When only those wells that exhibited an angiogenic response were included in this analysis, AI reflected the effect of the treatments on the growth of neovessels. These analyses were referred to as "pure angiogenic growth." The third measurement was also called the angiogenic index, but used all of the wells plated, including the wells that did not initiate an angiogenic response (zero values). Hence, these analyses were referred to as "overall angiogenic effect." Overall angiogenic effect reflected the combined effects of the first two measurements because it took into account the AI of all wells, whether or not they initiated an angiogenic response. Results were considered significant at the nominal level of 0.05 unless otherwise noted.

Initiation was compared between the control group and the treatment groups using a χ^2 test of association. If the overall table value was significant, pair-wise comparisons of the treatment groups to the control group were performed using either a χ^2 test of association or Fisher exact test with Bonferroni correction factors applied to the nominal level to maintain an overall type I error rate of 0.05.

Analysis of variance (ANOVA) was used to test for differences among group means for the overall angiogenic effect data. If the

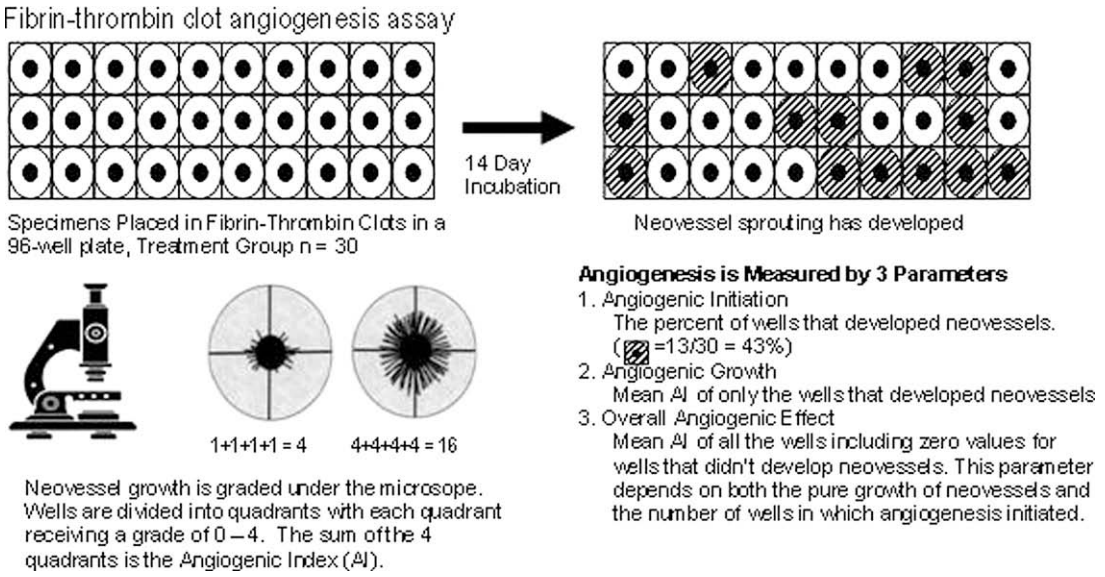

FIG. 1. Fibrin-thrombin clot angiogenesis assay. This figure depicts the setup of a typical 96-well plate for one treatment (30 wells). It also outlines the evaluation of angiogenesis microscopically and defines the angiogenic parameters used.

F test was significant, Dunnett's test was used to make *post hoc* pair-wise comparisons between the means of the control group and the treatment groups. Because of the reduction in sample sizes resulting from the exclusion of wells that did not exhibit an angiogenic response, the nonparametric Kruskal-Wallis test was used to check for differences among group means for the out zeros data. If the overall test was significant, pair-wise comparisons of the treatment groups to the control group were performed using the Wilcoxon rank-sum test with Bonferroni correction factors applied to the nominal level to maintain an overall type I error rate of 0.05.

Drugs and Drug Treatments

The 165-amino acid isoform of human recombinant vascular endothelial growth factor-A (VEGF) was obtained commercially from BioSource International (Camarillo, CA). Bevacizumab, a humanized monoclonal antibody to VEGF, was obtained as a kind gift from Genentech, Inc. (South San Francisco, CA). Human monoclonal antibodies to VEGFR-1 (IMC-18F1) and VEGFR-2 (IMC-1121) were kind gifts from IMClone Systems, Inc. (New York, NY). PTK787/ZK 222584 (PTK/ZK), a tyrosine kinase inhibitor of VEGF receptors, was a kind gift from Novartis AG (Basel, Switzerland). All drugs were reconstituted and stored according to manufacturers' recommendations.

VEGF-Stimulated Growth in Cultured Cells

Human umbilical vein endothelial cells (HUVEC) were obtained from ATCC (Rockville, MD) and propagated in the suggested medium (Hams F-12 Kaighns supplemented with 10% fetal bovine serum, antibiotic/antimycotic (Gibco, Grand Island, NY), 0.1 mg/mL heparin and 0.03 mg/mL endothelial growth supplement form bovine pituitary (Sigma, St. Louis MO) using gelatin-coated T-75 flasks (Becton and Dickson, Bedford, MA). For the experiment, cells were harvested and plated at 150,000 cells per T-75 flask in M-199 with 0.1% bovine serum albumin (control) or M-199 with 0.1% bovine serum albumin and 10 ng/mL rhVEGF (Biosource, Camarillo CA). Cells were counted at various time points (1 h [time zero], 20, 48, and 72 h) post-plating. One mm fields were randomly selected across the plate and cells within that area were counted (10× magnification). The sum of the 20 fields was used as an estimate of cell number for each time period. Data are reported as the percent change in estimated cell number from time zero.

RESULTS

VEGF was tested against the vein discs of five separate human placentas. Three different concentrations of VEGF were tested ranging from 1 to 100 ng/mL. VEGF-treated vein discs had more angiogenic initiation compared with control in none (0/5, 0%) of the placentas tested. VEGF-treated vein discs demonstrated more pure growth than control in 1 (20%) of five placentas. This was observed at only one concentration (1 ng/mL). VEGF treatment did not stimulate the overall angiogenic effect of vein discs in any (0/5, 0%) of the placentas tested (Table 1A).

Fragments of four separate malignant carcinoids were used to test VEGF at four different concentrations ranging from 1 to 1000 ng/mL. VEGF-treated carcinoid tumor fragments had more angiogenic initiation than control in 1 (50%) of 2 of the specimens tested with 10 ng/mL. One (50%) of 2 specimens tested with 10 ng/mL demonstrated a greater overall angiogenic effect as well. VEGF-treated carcinoid fragments failed to demonstrate more pure growth than control in any (0/4, 0%) of the tested specimens (Table 1B).

Three different concentrations of VEGF ranging from 1 to 100 ng/mL were tested against the fragments of four separate colon cancers. VEGF-treated colon cancer fragments had more angiogenic initiation compared with control in none (0/4, 0%) of the tumors tested. VEGF-treated colon cancer demonstrated more pure growth in one (33%) of three and one (25%) of four specimens at one and 10 ng/mL, respectively. These same VEGF-treated fragments experienced an increased overall angiogenic effect as well (Table 1C). In order to demonstrate bioactivity of the VEGF preparation,

TABLE 1

Effects of VEGF on Placental Vein Discs, Carcinoid Tumors, and Colon Cancer

(A) Effects of VEGF on Placental Vein Discs*

% of wells that initiated an angiogenic response†			
Concentration (ng/mL)	Inhibition‡	No effect§	Stimulation‖
1	1 of 5 (20%)	4 of 5 (80%)	0 of 5 (0%)
10	1 of 5 (20%)	4 of 5 (80%)	0 of 5 (0%)
10 w/o serum	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
100	1 of 5 (20%)	4 of 5 (80%)	0 of 5 (0%)
Pure growth of angiogenic blood vessels¶			
Concentration (ng/mL)	Inhibition‡	No effect§	Stimulation‖
1	1 of 5 (20%)	3 of 5 (60%)	1 of 5 (20%)
10	0 of 5 (0%)	5 of 5 (100%)	0 of 5 (0%)
10 w/o serum	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
100	0 of 5 (0%)	5 of 5 (100%)	0 of 5 (0%)
Overall angiogenic effect#			
Concentration (ng/mL)	Inhibition‡	No effect§	Stimulation‖
1	1 of 5 (20%)s	4 of 5 (80%)	0 of 5 (0%)
10	2 of 5 (40%)	3 of 5 (60%)	0 of 5 (0%)
10 w/out serum	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
100	1 of 5 (20%)	4 of 5 (80%)	0 of 5 (0%)

(B) Effects of VEGF on Carcinoid Tumors**

% of wells that initiated an angiogenic response†			
Concentration (ng/mL)	Inhibition‡	No effect§	Stimulation‖
1	0 of 2 (0%)	2 of 2 (100%)	0 of 2 (0%)
10	0 of 2 (0%)	1 of 2 (50%)	1 of 2 (50%)
100	0 of 4 (0%)	4 of 4 (100%)	0 of 4 (0%)
1000	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
Pure growth of angiogenic blood vessels¶			
Concentration (ng/mL)	Inhibition‡	No effect§	Stimulation‖
1	0 of 2 (0%)	2 of 2 (100%)	0 of 2 (0%)
10	0 of 2 (0%)	2 of 2 (100%)	0 of 2 (0%)
100	0 of 4 (0%)	4 of 4 (100%)	0 of 4 (0%)
1000	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
Overall angiogenic effect#			
Concentration (ng/mL)	Inhibition‡	No effect§	Stimulation‖
1	0 of 2 (0%)	2 of 2 (100%)	0 of 2 (0%)
10	0 of 2 (0%)	1 of 2 (50%)	1 of 2 (50%)
100	0 of 4 (0%)	4 of 4 (100%)	0 of 4 (0%)
1000	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)

(C) Effects of VEGF on the Colon Cancer††

% of wells that initiated an angiogenic response[2]			
Concentration (ng/mL)	Inhibition‡	No effect§	Stimulation‖
1	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
10	0 of 4 (0%)	4 of 4 (100%)	0 of 4 (0%)
100	0 of 4 (0%)	4 of 4 (100%)	0 of 4 (0%)
Growth of angiogenic blood vessels¶			
Concentration (ng/mL)	Inhibition‡	No effect§	Stimulation‖
1	0 of 3 (0%)	2 of 3 (67%)	1 of 3 (33%)
10	0 of 4 (0%)	3 of 4 (75%)	1 of 4 (25%)
100	0 of 4 (0%)	4 of 4 (100%)	0 of 4 (0%)
Overall angiogenic effect#			
Concentration (ng/mL)	Inhibition‡	No effect§	Stimulation‖
1	0 of 3 (0%)	2 of 3 (67%)	1 of 3 (33%)
10	0 of 4 (0%)	3 of 4 (75%)	1 of 4 (25%)
100	0 of 4 (0%)	4 of 4 (100%)	0 of 4 (0%)

*Data from five placentas, 30 wells per treatment for each placenta.

†Indicates the number of wells demonstrating an angiogenic response compared with the total number of wells prepared.

HUVEC were treated with VEGF at 10 ng/mL for up to 3 d. Cell numbers were estimated by counting multiple fields of the plated cells at various time periods. At 72 h, a 56% increase in cell number was observed in cells treated with VEGF compared with a 24% increase in non-treated cells (Fig. 2).

Bevacizumab was tested against the vein discs of three separate human placentas. Six different concentrations of this reagent were tested ranging from 0.01 to 1000 μg/mL. Bevacizumab-treated vein discs had less angiogenic initiation than control in none (0/3, 0%) and less angiogenic pure growth in none (0/3, 0%) of the placentas tested. While bevacizumab treatment inhibited the overall angiogenic effect in 1 (33%) of three placentas, this was only observed at one concentration (0.01 μg/mL) (Table 2A).

A single supraphysiologic concentration of bevacizumab (6.711 μM) was tested against seven carcinoids, five gynecologic tumors, and three additional human malignancies grouped together as "other" tumors. Bevacizumab-treated carcinoid fragments had less angiogenic initiation than control in four (57%) of seven the specimens tested. Bevacizumab-treated carcinoid fragments had less pure angiogenic growth than control in one (14%) of seven specimens tested. Bevacizumab treatment inhibited the overall angiogenic effect in five (71%) of seven carcinoids tested, one (20%) of five gynecologic tumors tested, and none (0/3, 0%) of the "other" tumors tested (Table 2B).

Both IMC-18F1 and IMC-1121 were tested against the vein discs of three separate human placentas. Four different concentrations of these compounds were tested ranging from 4 to 400 nM. IMC-18F1 and IMC-1121 treatment of vein discs yielded less angiogenic initiation than control in none (0/3, 0%) and less pure angiogenic growth in none (0/3, 0%) of the placentas tested. IMC-18F1 inhibited the overall angiogenic effect in none (0/3, 0%) of the placentas tested;

‡Inhibition indicates the number of specimens treated with VEGF that demonstrated statistically significant inhibition compared with control values.

§No effect indicates the number of specimens treated with VEGF that demonstrated no statistically significant difference from control values

‖Stimulation indicates the number of specimens treated with VEGF that demonstrated statistically significant growth compared with control values.

¶The angiogenic index only from wells that demonstrated an angiogenic response.

#The angiogenic index from all wells, whether or not an angiogenic response was present.

**Data from four carcinoid specimens, 30 wells per treatment for each specimen.

††Data from four colon cancer specimens, 30 wells per treatment for each specimen.

IMC-1121 inhibited the overall angiogenic effect in 1 (33%) of three placentas tested, at only one concentration (40 nM) (Table 3A).

Three different concentrations of both IMC-18F1 and IMC-1121 ranging from 1 to 100 nM were tested against fragments of three separate malignant carcinoids. Both IMC-18F1 and IMC-1121 treatment of carcinoids yielded less angiogenic initiation than control in none (0/3, 0%) of the specimens tested. IMC-18F1 treatment of carcinoids yielded less pure angiogenic growth in none (0/3, 0%) of the specimens tested, while IMC-1121 yielded less pure angiogenic growth in one (33%) of three carcinoids tested. Both IMC-18F1 and IMC-1121 inhibited the overall angiogenic effect in none (0/3, 0%) of the carcinoids tested (Table 3B).

PTK/ZK was tested against vein discs from eight separate human placentas. Eleven different concentrations of PTK/ZK were tested ranging from 0.1 to 100 μM. PTK/ZK-treated vein discs had less angiogenic initiation than control in all (100%) of the placentas tested at six of the 11 different concentrations. PTK/ZK-treated vein discs had less pure angiogenic growth in all (100%) of the placentas tested at two different concentrations. PTK/ZK treatment inhibited the overall angiogenic effect in all (100%) of the tested placentas at nine of the 11 concentrations tested (Table 4A).

Fragments of 21 separate gynecological tumors were used to test PTK/ZK. Ten different concentrations of PTK/ZK were tested ranging from 1 to 100 μM. PTK/ZK-treated gynecologic tumors had less angiogenic initiation than control in at least one specimen at all 10 concentrations tested; 50 μM of PTK/ZK yielded less angiogenic initiation in five (50%) of the 10 specimens tested. PTK-treated tumors had less pure angiogenic growth in at least one specimen at six different concentrations; 25 μM of PTK/ZK yielded less angiogenic growth in three (38%) of the eight specimens tested. PTK/ZK treatment inhibited the overall angiogenic effect in at least one specimen in all 10 concentrations tested. At eight of 10 concentrations, PTK/ZK treatment inhibited the overall angiogenic effect in at least 50% of specimens (Table 4B).

Twelve concentrations of PTK/ZK ranging from 1 to 100 μM were tested against the fragments of 15 separate carcinoids. PTK-treated carcinoid tumors had less angiogenic initiation than control in at least one specimen at nine of 12 concentrations tested; 25 μM of PTK/ZK yielded less angiogenic initiation in three (75%) of the four specimens tested. PTK-treated tumors had less pure angiogenic growth in at least one specimen at four different concentrations; 20 μM of PTK/ZK yielded less angiogenic growth in three (33%) of the nine specimens tested. PTK/ZK treatment inhibited the overall angiogenic effect in at least 50% of tested specimens at all 12 concentrations tested (Table 4C).

PTK/ZK was tested against the fragments of 10 tumors categorized as "other." Eight concentrations of PTK/ZK ranging from 5 to 80 μM were tested. PTK-treated "other" tumors had less angiogenic initiation than control in at two (25%) of eight specimens at one concentration (40 μM). PTK-treated tumors had less pure angiogenic growth in one specimen at four different concentrations. PTK/ZK treatment inhibited the overall angiogenic effect in at least one specimen in six of eight concentrations tested. At three of eight concentrations, PTK/ZK treatment inhibited the overall angiogenic effect in at least 50% of specimens (Table 4D).

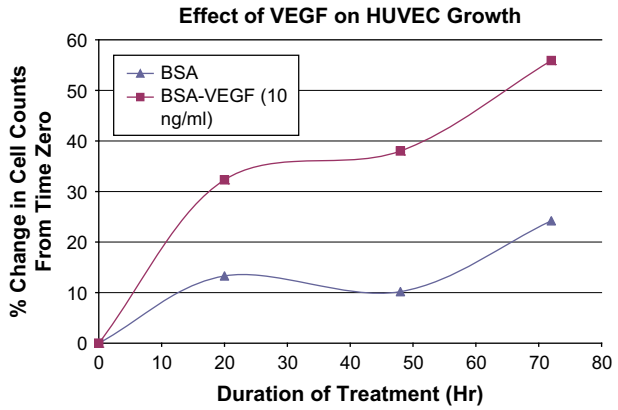

FIG. 2. Effect of VEGF on HUVEC growth. Attached HUVEC cells were maintained for 72 h in serum-depleted culture media consisting of M-199 and 0.1% BSA (control; —△—) or consisting of the control medium plus 10ng/ml VEGF (—■—). Cells were counted in 20 randomly identified 1mm areas and summed. The data are expressed as the change in cell counts from time zero. Over 72 h HUVEC growth was consistently greater in VEGF-treated plates than in the control plates.

DISCUSSION

Countless experiments have implicated VEGF in angiogenesis. Among the first to do this were Ferrara and Henzel [5]. They observed that VEGF-stimulated HUVEC proliferation was more than 300% greater than that of control cells. Congruent data presented around the same time by Connolly *et al.* [6], Plouët *et al.* [16], Senger [17], and others corroborated this observation. In 1992, Pepper *et al.* observed that administration of VEGF to bovine endothelial cells grown on a collagen gel induced the cells to invade the collagen and form 3-D, capillary-like tubules [4].

In vivo, VEGF has stimulated neovessel formation in the chorioallantoic membrane [18], in both rat [6], and rabbit corneas [19] as well as matrigel models [20]. Montrucchio *et al.* showed that the mean area percent of blood vessels in VEGF-containing matrigels was more

TABLE 2

Effects of Bevacizumab on Placental Vein Discs and Tumors

(A) Effects of Bevacizumab on Placental Vein Discs*

% of wells that initiated an angiogenic response†			
Concentration (μg/mL)	Inhibition‡	No effect§	Stimulation‖
0.01	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
0.1	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
1	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
10	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
100	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
1000	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
Growth of angiogenic blood vessels¶			
Concentration (μg/mL)	Inhibition‡	No effect§	Stimulation‖
0.01	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
0.1	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
1	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
10	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
100	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
1000	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
Overall angiogenic effect#			
Concentration (μg/mL)	Inhibition‡	No effect§	Stimulation‖
0.01	1 of 3 (33%)	2 of 3 (66%)	0 of 3 (0%)
0.1	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
1	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
10	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
100	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
1000	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)

(B) Effects of Bevacizumab on Tumors**

% of wells that initiated an angiogenic response†			
Tumor type	Inhibition‡	No effect§	Stimulation‖
Gynecologic	0 of 5 (0%)	5 of 5 (100%)	0 of 5 (0%)
Carcinoid	4 of 7 (57%)	3 of 7 (43%)	0 of 7 (0%)
Other	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
Growth of angiogenic blood vessels¶			
Tumor type	Inhibition‡	No effect§	Stimulation‖
Gynecologic	0 of 5 (0%)	5 of 5 (100%)	0 of 5 (0%)
Carcinoid	1 of 7 (14%)	6 of 7 (86%)	0 of 7 (0%)
Other	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
Overall angiogenic effect#			
Tumor type	Inhibition‡	No Effect§	Stimulation‖
Gynecologic	1 of 5 (20%)	4 of 5 (80%)	0 of 5 (0%)
Carcinoid	5 of 7 (71%)	2 of 7 (29%)	0 of 7 (0%)
Other	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)

*Data from three placentas, 30 wells per treatment for each placenta.

†Indicates the number of wells demonstrating an angiogenic response compared with the total number of wells prepared.

‡Inhibition indicates the number of specimens treated with bevacizumab that demonstrated statistically significant inhibition compared with control values.

§No effect indicates the number of specimens treated with bevacizumab that demonstrated no statistically significant difference from control values.

‖Stimulation indicates the number of specimens treated with bevacizumab that demonstrated statistically significant growth compared with control values.

¶The angiogenic index only from wells that demonstrated an angiogenic response.

#The angiogenic index from all wells, whether or not an angiogenic response was present.

**Data from five gynecologic tumors, seven carcinoid specimens, and three separate "other" tumors treated with 6.7 μM bevacizumab, 30 wells per treatment for each specimen.

than 10 times that of control matrigels [20]. Takeshita *et al.* induced ischemia in rabbit hind limbs by excising a femoral artery [21]. When 500-1000 μg VEGF was administered as a single bolus in the animal's ipsilateral iliac artery, statistically greater blood pressure, higher angiographic scores, and greater histologic capillary density than in control ischemic limbs were observed. Primate neovessel stimulation was demonstrated when Tolentino *et al.* observed neovascularization in cytomologous monkey eyes injected with VEGF [22].

A causative role for VEGF in angiogenesis has been further supported through inhibition studies. Bevacizumab, a monoclonal antibody to VEGF-A, has been tested against numerous human tumor xenografts. A recent review by Gerber and Ferrara [7] outlined the results of 30 different experiments in which an anti-VEGF monoclonal antibody ligand was given to inhibit tumor growth in nude mice with human tumor xenografts. These 30 studies accounted for 12 different tumor types. Tumor growth inhibition ranged from 25% to 95% compared with control mice of the same tumor types. In 15 experiments, neovascularization was also notably less in the tumors following anti-VEGF treatment.

Reagents that target the VEGF receptors have also been shown to inhibit tumor growth in animals. IMC-18F1, a human neutralizing antibody inhibiting VEGF/VEGFR-1, given intraperitoneally to athymic nude mouse xenografts significantly suppressed tumor growth of human breast cancer [8]. IMC-1121, a humanized anti-kdr antibody, inhibited the mitogenesis of VEGF-stimulated HUVECs with an ED_{50} of 0.7nM. One hundred micrograms of this reagent given intraperitoneally significantly increased the survival of NOD-SCID mice inoculated with HL60 leukemia cells [9].

In humans, there have been many reports of VEGF's overexpression in proangiogenic settings. Nissen *et al.* showed that VEGF was much more prevalent in surgical wound serum than in normal serum [11]. Shifren *et al.* observed a direct correlation between the amount of endometrial VEGF mRNA and the degree of neovascularization occurring in the human menstrual cycle [12]. Yamamoto *et al.* found that VEGF levels in human serum were higher in patients harboring an active malignancy and that it correlated positively to the stage of disease in breast cancer [23]. Despite these and other reports of temporal and spatial up-regulation of VEGF in settings of increased vascularity, there are scant data that describe VEGF directly stimulating neovascularization in humans. The VIVA trial was a randomized controlled trial attempting to provide therapeutic angiogenesis to patients with stable exertional angina [24]. One hundred seventy-eight patients were randomized to receive either intra-arterial rh-

TABLE 3

Effects of IMC-18F1 and IMC-1121 on Placental Vein Discs and Carcinoid Tumors

(A) Effects of IMC-18F1 and IMC-1121 on Placental Vein Discs*

% of wells that initiated an angiogenic response†			
Reagent (nM)	Inhibition‡	No effect§	Stimulation‖
IMC-18F1 (4)	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
IMC-18F1 (10)	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
IMC-18F1 (40)	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
IMC-18F1 (100)	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
IMC-18F1 (400)	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
IMC-1121 (4)	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
IMC-1121 (10)	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
IMC-1121 (40)	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
IMC-1121 (100)	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
IMC-1121 (400)	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
Growth of angiogenic blood vessels¶			
Reagent (nM)	Inhibition‡	No effect§	Stimulation‖
IMC-18F1 (4)	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
IMC-18F1 (10)	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
IMC-18F1 (40)	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
IMC-18F1 (100)	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
IMC-18F1 (400)	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
IMC-1121 (4)	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
IMC-1121 (10)	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
IMC-1121 (40)	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
IMC-1121 (100)	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
IMC-1121 (400)	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
Overall angiogenic effect#			
Reagent (nM)	Inhibition‡	No effect§	Stimulation‖
IMC-18F1 (4)	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
IMC-18F1 (10)	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
IMC-18F1 (40)	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
IMC-18F1 (100)	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
IMC-18F1 (400)	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
IMC-1121 (4)	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
IMC-1121 (10)	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
IMC-1121 (40)	1 of 3 (33%)	2 of 3 (67%)	0 of 3 (0%)
IMC-1121 (100)	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
IMC-1121 (400)	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)

(B) Effects of IMC-18F1 and IMC-1121 on Carcinoids**

% of wells that initiated an angiogenic response†			
Reagent (nM)	Inhibition‡	No effect§	Stimulation‖
IMC-18F1 (1)	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
IMC-18F1 (10)	0 of 3 (0%)	2 of 3 (67%)	1 of 3 (33%)
IMC-18F1 (100)	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
IMC-1121 (1)	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
IMC-1121 (10)	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
IMC-1121 (100)	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
Growth of angiogenic blood vessels¶			
Reagent (nM)	Inhibition‡	No Effect§	Stimulation‖
IMC-18F1 (1)	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
IMC-18F1 (10)	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
IMC-18F1 (100)	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
IMC-1121 (1)	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
IMC-1121 (10)	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
IMC-1121 (100)	1 of 3 (33%)	2 of 3 (67%)	0 of 3 (0%)
Overall angiogenic effect#			
Reagent (nM)	Inhibition‡	No effect§	Stimulation‖
IMC-18F1 (1)	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
IMC-18F1 (10)	0 of 3 (0%)	2 of 3 (67%)	1 of 3 (33%)
IMC-18F1 (100)	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
IMC-1121 (1)	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
IMC-1121 (10)	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
IMC-1121 (100)	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)

(*Continued*)

TABLE 3

Effects of IMC-18F1 and IMC-1121 on Placental Vein Discs and Carcinoid Tumors (*Continued*)

(B) Effects of IMC-18F1 and IMC-1121 on Carcinoids**

Overall angiogenic effect#			
IMC-1121 (10)	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
IMC-1121 (100)	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)

*Data from three placentas, 30 wells per treatment for each placenta.

†Indicates the number of wells demonstrating an angiogenic response compared with the total number of wells prepared.

‡Inhibition indicates the number of specimens treated with IMC-18F1 or IMC-1121 that demonstrated statistically significant inhibition compared with control values.

§No Effect indicates the number of specimens treated with IMC-18F1 or IMC-1121 that demonstrated no statistically significant difference from control values.

‖Stimulation indicates the number of specimens treated with IMC-18F1 or IMC-1121 that demonstrated statistically significant growth compared with control values.

¶The angiogenic index only from wells that demonstrated an angiogenic response.

#The angiogenic index from all wells, whether or not an angiogenic response was present.

**Data from five carcinoid specimens, treated with IMC-18F1 or IMC-1121, 30 wells per treatment for each specimen.

VEGF-A_{165} or placebo. The primary endpoint of this trial was a significant change between treatment and placebo arms in treadmill time from baseline to day 60, and this was not observed. In addition, no significant improvements in summed rest or summed stress scores as quantified by myocardial perfusion studies were observed after 60 d. The fact that bevacizumab was the first "antiangiogenic" to improve survival in patients with malignant neoplasia implies that VEGF plays a role in human tumor angiogenesis [25]. However, bevacizumab has only been effective in select groups of patients and only when used in combination with chemotherapy. As a result, many have theorized that rather than frankly inhibiting blood vessel growth bevacizumab may stabilize aberrant tumor vessels facilitating more efficient delivery of cytotoxics to the tumor itself [26].

To determine if VEGF directly stimulates neovessel growth from human tissues, we designed a series of experiments using a fibrin-thrombin clot angiogenesis assay. We directly examined the growth of new blood vessels from architecturally intact human vein and tumor tissues. We compared the angiogenic response of these tissues when treated with VEGF-A_{165} to the angiogenic response of these tissues when treated with control media. We quantified the angiogenic response according to three criteria: the percentage of wells that develop neovessels, the degree of pure growth undertaken by these neovessels, and the combination of

TABLE 4

Effects of PTK/ZK on Placental Vein Discs, Gynecologic Tumors, and Carcinoid Tumors

(A) Effects of PTK/ZK on placental vein discs*

% of wells that initiated an angiogenic response†

Concentration (μM)	Inhibition‡	No effect§	Stimulation‖
0.1	0 of 1 (0%)	1 of 1 (100%)	0 of 1 (0%)
1	6 of 6 (100%)	0 of 6 (0%)	0 of 6 (0%)
10	1 of 3 (33%)	2 of 3 (67%)	0 of 3 (0%)
20	1 of 2 (59%)	1 of 2 (50%)	0 of 2 (0%)
25	1 of 3 (33%)	2 of 3 (67%)	0 of 3 (0%)
40	2 of 2 (100%)	0 of 2 (0%)	0 of 2 (0%)
50	3 of 3 (100%)	0 of 3 (0%)	0 of 3 (0%)
60	1 of 1 (100%)	0 of 1 (0%)	0 of 1 (0%)
75	3 of 3 (100%)	0 of 3 (0%)	0 of 3 (0%)
80	1 of 1 (100%)	0 of 1 (0%)	0 of 1 (0%)
100	0 of 4 (0%)	4 of 4 (100%)	0 of 4 (0%)

Growth of angiogenic blood vessels¶

Concentration (μM)	Inhibition‡	No effect§	Stimulation‖
0.1	0 of 1 (0%)	1 of 1 (100%)	0 of 1 (0%)
1	NI		
10	2 of 3 (67%)	1 of 3 (33%)	0 of 3 (0%)
20	2 of 2 (100%)	0 of 2 (0%)	0 of 2 (0%)
25	1 of 3 (33%)	2 of 3 (67%)	0 of 3 (0%)
40	2 of 2 (100%)	0 of 2 (0%)	0 of 2 (0%)
50	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
60	0 of 1 (0%)	1 of 1 (100%)	0 of 1 (0%)
75	0 of 2 (0%)	2 of 2 (100%)	0 of 2 (0%)
80	NI		
100	0 of 4 (0%)	4 of 4 (100%)	0 of 4 (0%)

Overall angiogenic effect#

Concentration (μM)	Inhibition‡	No effect§	Stimulation‖
0.1	0 of 1 (0%)	1 of 1 (100%)	0 of 1 (0%)
1	6 of 6 (100%)	0 of 6 (0%)	0 of 6 (0%)
10	3 of 3 (100%)	0 of 3 (0%)	0 of 3 (0%)
20	2 of 2 (100%)	0 of 2 (0%)	0 of 2 (0%)
25	3 of 3 (100%)	0 of 3 (0%)	0 of 3 (0%)
40	2 of 2 (100%)	0 of 2 (0%)	0 of 2 (0%)
50	3 of 3 (100%)	0 of 3 (0%)	0 of 3 (0%)
60	1 of 1 (100%)	0 of 1 (0%)	0 of 1 (0%)
75	3 of 3 (100%)	0 of 3 (0%)	0 of 3 (0%)
80	1 of 1 (100%)	0 of 1 (0%)	0 of 1 (0%)
100	0 of 4 (0%)	4 of 4 (100%)	0 of 4 (0%)

(B) Effects of PTK/ZK on Gynecologic Tumors**

% of wells that initiated an angiogenic response†

Concentration (μM)	Inhibition‡	No effect§	Stimulation‖
1	2 of 8 (25%)	6 of 8 (75%)	0 of 8 (0%)
5	1 of 9 (11%)	8 of 9 (89%)	0 of 9 (0%)
10	2 of 12 (17%)	10 of 12 (87%)	0 of 12 (0%)
15	1 of 5 (20%)	4 of 5 (80%)	0 of 5 (0%)
20	1 of 6 (17%)	5 of 6 (83%)	0 of 6 (0%)
25	4 of 9 (44%)	5 of 9 (56%)	0 of 9 (0%)
40	3 of 11 (27%)	8 of 11 (73%)	0 of 11 (0%)
50	5 of 10 (50%)	5 of 10 (50%)	0 of 10 (0%)
80	1 of 1 (100%)	0 of 1 (0%)	0 of 1 (0%)
100	2 of 10 (20%)	8 of 10 (80%)	0 of 10 (0%)

Growth of angiogenic blood vessels¶

Concentration (μM)	Inhibition‡	No effect§	Stimulation‖
1	0 of 4 (0%)	4 of 4 (100%)	0 of 4 (0%)
5	1 of 8 (13%)	7 of 8 (87%)	0 of 8 (0%)
10	1 of 10 (10%)	9 of 10 (90%)	0 of 10 (0%)
15	2 of 4 (50%)	2 of 4 (50%)	0 of 4 (0%)

(*Continued*)

TABLE 4

Effects of PTK/ZK on Placental Vein Discs, Gynecologic Tumors, and Carcinoid Tumors (*Continued*)

(B) Effects of PTK/ZK on Gynecologic Tumors**

20	0 of 4 (0%)	4 of 4 (100%)	0 of 4 (0%)
25	3 of 8 (38%)	5 of 8 (62%)	0 of 8 (0%)
40	1 of 6 (27%)	5 of 6 (83%)	0 of 6 (0%)
50	1 of 6 (17%)	4 of 6 (67%)	1 of 6 (17%)
80	NI		
100	0 of 10 (0%)	9 of 10 (90%)	1 of 10 (10%)

Overall angiogenic effect#

Concentration (μM)	Inhibition‡	No effect§	Stimulation‖
1	3 of 8 (38%)	5 of 8 (62%)	0 of 8 (0%)
5	6 of 9 (67%)	3 of 9 (33%)	0 of 9 (0%)
10	8 of 12 (67%)	4 of 12 (33%)	0 of 12 (0%)
15	4 of 5 (80%)	1 of 5 (20%)	0 of 5 (0%)
20	4 of 6 (67%)	2 of 6 (33%)	0 of 6 (0%)
25	7 of 9 (78%)	2 of 9 (22%)	0 of 9 (0%)
40	8 of 11 (73%)	3 of 11 (27%)	0 of 11 (0%)
50	5 of 10 (50%)	5 of 10 (50%)	0 of 10 (0%)
80	1 of 1 (100%)	0 of 1 (0%)	0 of 1 (0%)
100	1 of 10 (10%)	9 of 10 (90%)	0 of 10 (0%)

(C) Effects of PTK/ZK on Carcinoid Tumors††

% of wells that initiated an angiogenic response†

Concentration (μM)	Inhibition‡	No effect§	Stimulation‖
1	1 of 1 (100%)	0 of 1 (0%)	0 of 1 (0%)
5	2 of 12 (17%)	10 of 12 (83%)	0 of 12 (0%)
10	4 of 14 (29%)	10 of 14 (71%)	0 of 14 (0%)
15	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
20	2 of 11 (18%)	9 of 11 (82%)	0 of 11 (0%)
25	3 of 4 (75%)	1 of 4 (25%)	0 of 4 (0%)
40	5 of 14 (36%)	9 of 14 (64%)	0 of 14 (0%)
50	1 of 4 (25%)	3 of 4 (75%)	0 of 4 (0%)
60	0 of 1 (0%)	1 of 1 (100%)	0 of 1 (0%)
75	1 of 1 (100%)	0 of 1 (0%)	0 of 1 (0%)
80	1 of 1 (100%)	0 of 1 (0%)	0 of 1 (0%)
100	0 of 1 (0%)	1 of 1 (100%)	0 of 1 (0%)

Growth of angiogenic blood vessels¶

Concentration (μM)	Inhibition‡	No effect§	Stimulation‖
1	NI		
5	4 of 11 (36%)	7 of 11 (64%)	0 of 11 (0%)
10	3 of 13 (23%)	10 of 13 (77%)	0 of 13 (0%)
15	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
20	3 of 9 (33%)	6 of 9 (67%)	0 of 9 (0%)
25	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
40	3 of 10 (30%)	7 of 10 (70%)	0 of 10 (0%)
50	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
60	0 of 1 (0%)	1 of 1 (100%)	0 of 1 (0%)
75	NI		
80	0 of 1 (0%)	1 of 1 (100%)	0 of 1 (0%)
100	0 of 1 (0%)	1 of 1 (100%)	0 of 1 (0%)

Overall angiogenic effect#

Concentration (μM)	Inhibition‡	No effect§	Stimulation‖
1	1 of 1 (100%)	0 of 1 (0%)	0 of 1 (0%)
5	8 of 12 (67%)	4 of 12 (33%)	0 of 12 (0%)
10	10 of 14 (71%)	4 of 14 (29%)	0 of 14 (0%)
15	3 of 3 (100%)	0 of 3 (0%)	0 of 3 (0%)
20	7 of 11 (64%)	4 of 11 (36%)	0 of 11 (0%)
25	4 of 4 (100%)	0 of 4 (0%)	0 of 4 (0%)
40	10 of 14 (71%)	4 of 14 (29%)	0 of 14 (0%)
50	2 of 4 (50%)	2 of 4 (50%)	0 of 4 (0%)
60	1 of 1 (100%)	0 of 1 (0%)	0 of 1 (0%)
75	1 of 1 (100%)	0 of 1 (0%)	0 of 1 (0%)

(*Continued*)

TABLE 4

Effects of PTK/ZK on Placental Vein Discs, Gynecologic Tumors, and Carcinoid Tumors (*Continued*)

(C) Effects of PTK/ZK on Carcinoid Tumors[††]

	Overall angiogenic effect[#]		
80	1 of 1 (100%)	0 of 1 (0%)	0 of 1 (0%)
100	1 of 1 (100%)	0 of 1 (0%)	0 of 1 (0%)

(D) Effects of PTK/ZK on Other Tumors[‡‡]

	% of wells that initiated an angiogenic response[†]		
Concentration (μM)	Inhibition[‡]	No effect[§]	Stimulation[‖]
5	0 of 5 (0%)	5 of 5 (100%)	0 of 5 (0%)
10	0 of 8 (0%)	8 of 8 (100%)	0 of 8 (0%)
15	0 of 3 (0%)	3 of 3 (100%)	0 of 3 (0%)
20	0 of 6 (0%)	6 of 6 (100%)	0 of 6 (0%)
25	0 of 2 (0%)	2 of 2 (100%)	0 of 2 (0%)
40	2 of 8 (25%)	6 of 8 (75%)	0 of 8 (0%)
50	0 of 4 (0%)	4 of 4 (100%)	0 of 4 (0%)
80	0 of 1 (0%)	1 of 1 (100%)	0 of 1 (0%)
	Growth of angiogenic blood vessels[¶]		
Concentration (μM)	Inhibition[‡]	No effect[§]	Stimulation[‖]
5	1 of 4 (25%)	3 of 4 (75%)	0 of 4 (0%)
10	1 of 6 (17%)	5 of 6 (83%)	0 of 6 (0%)
15	1 of 1 (100%)	0 of 1 (0%)	0 of 1 (0%)
20	0 of 4 (0%)	4 of 4 (100%)	0 of 4 (0%)
25	1 of 2 (50%)	1 of 2 (50%)	0 of 2 (0%)
40	0 of 5 (0%)	5 of 5 (100%)	0 of 5 (0%)
50	0 of 2 (0%)	2 of 2 (100%)	0 of 2 (0%)
80	NI		
	Overall angiogenic effect[#]		
Concentration (μM)	Inhibition[‡]	No effect[§]	Stimulation[‖]
5	2 of 5 (40%)	3 of 5 (60%)	0 of 5 (0%)
10	1 of 8 (12%)	7 of 8 (88%)	0 of 8 (0%)
15	2 of 3 (67%)	1 of 3 (33%)	0 of 3 (0%)
20	2 of 6 (33%)	4 of 6 (67%)	0 of 6 (0%)
25	2 of 2 (100%)	0 of 2 (0%)	0 of 2 (0%)
40	4 of 8 (50%)	4 of 8 (50%)	0 of 8 (0%)
50	0 of 4 (0%)	4 of 4 (100%)	0 of 4 (0%)
80	0 of 1 (0%)	1 of 1 (100%)	0 of 1 (0%)

[*]Data from eight placentas, 30 wells per treatment for each placenta.

[†]Indicates the number of wells demonstrating an angiogenic response compared with the total number of wells prepared.

[‡]Inhibition indicates the number of specimens treated with PTK/ZK that demonstrated statistically significant inhibition compared with control values.

[§]No Effect indicates the number of specimens treated with PTK/ZK that demonstrated no statistically significant difference from control values.

[‖]Stimulation indicates the number of specimens treated with PTK/ZK that demonstrated statistically significant growth compared with control values.

[¶]The angiogenic index only from wells that demonstrated an angiogenic response.

[#]The angiogenic index from all wells, whether or not an angiogenic response was present.

[**]Data from 21 gynecologic tumors treated with PTK/ZK, 30 wells per treatment for each specimen.

[††]Data from 12 carcinoid tumors treated with PTK/ZK, 30 wells per treatment for each specimen.

[‡‡]Data from nine "other" tumors, treated with PTK/ZK, 30 wells per treatment for each specimen.

these two parameters designated as the overall angiogenic effect. The concentrations of VEGF tested were consistent with those that have profoundly stimulated angiogenesis in other *in vitro* models (Table 5A). In our study, adding VEGF to vein discs stimulated only pure angiogenic growth in one of five placentas at only one concentration. VEGF treatment did not appreciably stimulate neovascular initiation, nor did it stimulate the overall angiogenic effect of any placental vein discs compared with control. The addition of VEGF to colon cancer fragments stimulated pure angiogenic growth in only one specimen at two separate concentrations, while the addition of VEGF to carcinoid fragments failed to stimulate pure angiogenic growth in any specimen. We then used the same lot and concentration (10 ng/mL) of VEGF to treat HUVECs; and we observed stimulation of cellular proliferation in the treated HUVECs as would be expected. We considered the possibility that the 20% serum we used could maximally stimulate the angiogenic response of these tissues, obviating any additional stimulatory effect that would be seen by adding VEGF to the system. However, we tested the vein discs of three placentas against VEGF in serum-free media, and stimulation of angiogenesis was still not observed (Table 1A). In summary, the addition of VEGF to human tissues in this angiogenic assay did not consistently stimulate neovascularization.

To further assess the role of the VEGF in human tissues, we performed a similar set of experiments testing reagents that inhibit different elements of the VEGF pathway. First, we tested bevacizumab which targets the VEGF-A ligand. This reagent had very minimal inhibitory effects on placental vein tissue and practically no effect on gynecologic tumors or on "other" tumors. Bevacizumab statistically inhibited the overall angiogenic effect of 5 of 7 (71%) carcinoid tumors. Next we tested two humanized monoclonal antibodies directed against VEGF receptors. IMC-18F1 targets VEGF/VEGFR-1 ligand binding while IMC-1121 blocks the VEGF/VEGFR-2 interaction [8, 9]. Neither of these reagents inhibited human angiogenesis in this model with any consistency. Finally, we tested PTK/ZK. This reagent is a tyrosine kinase inhibitor that targets all of the known VEGF receptors (VEGFR-1, -2, and -3) as well as other non-VEGF receptors [27]. PTK/ZK inhibited the overall angiogenic effect of vein discs in 100% of placentas tested at nine different concentrations. PTK/ZK inhibited the overall angiogenic effect of gynecologic tumor fragments in greater than 50% of specimens tested at eight different concentrations. In addition, PTK/ZK inhibited the overall angiogenic effect of carcinoid tumor fragments in greater than 50% of specimens tested at 12 different concentrations.

TABLE 5

In Vitro Profile of PTK/ZK and Commonly Tested Concentrations of VEGF

(A) Commonly Tested Concentrations of VEGF

Reagent	Our dose	In vitro dose	Animal dose	Human dose
VEGF	HPVAM .1–100 ng/mL Tumors 1–1000 ng/mL	20–100 ng [4-6]	250 ng–1.25μg [22]	17–50 ng/kg/min [24]
Bevacizumab	HPVAM = .01–1000 μg/mL Tumors = 1000 μg/mL (6.7 μM)	50 ng/mL [5]	175–340 μg/mL [30]	200–300 μg/mL [31]
IMC-18F1	HPVAM = 4–400 nM (.6–60 ug/mL) Tumors = 1–100 nM (.15–15 μg/mL)	54 pmol [8]	88 μg/mL [8]	NA
IMC-1121	HPVAM = 4–400 nM (.6–60 μg/mL) Tumors = 1–100 nM (.15–15μg/mL)	1 nM [9]	NA	>5 μg/mL [32]
PTK/ZK	HPVAM = 1–100 μM Tumors = 1–100 μM	100 nM [28]	30 μM [28]	13 μM [27]

(B) In Vitro Profile of PTK/ZK: Inhibitory Activity Against RTKs [28]

Kinase	IC_{50} (μM)
VEGF-R1/Flt-1	0.077
VEGF-R2/KDR	0.037
VEGF-R2/Flk	0.27
VEGF-R3/Flt-4	0.66
PDGF-R-β	0.58
c-Kit	0.73
c-Fms	1.4

PTK/ZK inhibits VEGFR-1 and VEGFR-2 with greatest potency, but it also effects VEGFR-3 and other protein tyrosine kinases including PDGFR, c-kit, and c-FMS (Table 5B). Wood *et al.* observed inhibition of VEGF-stimulated proliferation and migration of HUVECs with doses of PTK/ZK less than 100 nM [28]. Oral administration of 50 to 100 mg/kg of PTK/ZK successfully impaired tumor growth in both human prostate and colon cancer xenografts. Studies of PTK/ZK in humans have demonstrated an oral dose of 1000 mg to be safe [27], and the steady state plasma concentration (1–15 μM) achieved with this dose is consistent with the range of concentrations we tested here (1–80 μM). Although the preliminary results from a recent Phase III trial failed to show any improvements in overall 1-y survival for all patients with stage 4 colorectal cancer treated with PTK/ZK and FOLFOX4 [29], a subset of patients with elevated LDH levels (>1.5 X ULN) showed a modest survival advantage by the addition of PTK/ZK to their chemotherapy regimen. Furthermore, in the authors' personal clinical experience with this reagent, PTK/ZK has been a useful monotherapy in selected patients suffering from neuroendocrine tumors (data not shown).

In summary, VEGF did not consistently stimulate neovascularization in human veins or in malignant tumor fragments. Antibodies targeting VEGFR-1 and VEGFR-2 did not inhibit neovascularization in human tissues. Bevacizumab, while not affecting placental veins or gynecologic tumors, did moderately inhibit angiogenesis in the subset of carcinoid tumors. PTK/ZK inhibited angiogenesis in every tissue type tested at multiple different concentrations.

Bevacizumab blocks the VEGF-A ligand directly, and this should theoretically enable it to affect a broader spectrum of downstream targets than reagents that affect only VEGFR-1 or VEGFR-2. This distinction may explain why bevacizumab was able to inhibit angiogenesis in carcinoid tumors while IMC-18F1 and IMC-1121 did not. In the same way, PTK/ZK affects many more targets than bevacizumab, influencing VEGF, PDGF, and other non-VEGF pathways. This distinction, we feel, explains why the inhibitory effect of PTK/ZK was much more consistent and profound than the other reagents tested.

We recognize the limitations of any *in vitro* assay model. However, these results suggest that in human tissues, simply stimulating or blocking the VEGF pathway alone does not consistently alter neovascularization. The role of VEGF in human angiogenesis appears to be complex, maintaining varying degrees of importance according to tissue type. Manipulations of human neovascularization appear to be more consistently achieved when multiple growth factor pathways are affected.

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