Roles of Nitric Oxide Synthase Inhibition and Vascular Endothelial Growth Factor Receptor-2 Inhibition on Vascular Morphology and Function in an In vivo Model of Pancreatic Cancer

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Abstract

Purpose: Both nitric oxide (NO) and vascular endothelial growth factor (VEGF) mediate tumor vascular function. Because these molecules regulate one another’s expression, we hypothesized that NO synthase (NOS) inhibition produces effects comparable to those of anti-VEGF therapy on human pancreatic cancer xenografts.

Experimental Design: L3.6pl human pancreatic cancer cells were s.c. implanted in nude mice. On day 6, mice were randomized to receive (a) PBS (control), (b) DC101 [VEGF receptor 2 (VEGFR-2) antibody] by i.p. injection, (c) N-nitro-L-arginine (NNLA; NOS inhibitor) in the drinking water, or (d) both DC101 and NNLA. Mice were killed on day 20.

Results: DC101 and NNLA as single agents inhibited tumor growth by ~50% to 60% (P < 0.008 for both). Furthermore, combined therapy inhibited mean tumor growth by 89% (P < 0.008). Combined inhibition of VEGFR-2 and NOS also decreased mean vessel counts by 65% (P < 0.03) and vessel area by 80% versus controls (P < 0.001). In contrast to DC101 where vessel diameter was similar to control, NNLA decreased mean vessel diameter by 42% (P < 0.001). NNLA also led to a 54% (P < 0.03) decrease in tumor uptake of the perfusion marker Hoechst 33342 versus controls whereas DC101 decreased Hoechst 33342 staining by 43% (P < 0.03). The combination of inhibitors decreased perfusion by 73% (P < 0.03).

Conclusions: Although VEGFR-2 can mediate NOS activity, the combination of VEGFR-2 and NOS inhibition significantly increased the antivasculareffect oversingle agent therapy. The addition of NOS inhibition led to an even further alteration of tumor vessel morphology and vascular perfusion compared with VEGFR-2 blockade, suggesting that NO and VEGFR-2 have distinct but complementary effects on the tumor vasculature.
and function in a model of pancreatic cancer. Although we found that both NOS inhibition and VEGF receptor 2 (VEGFR-2) blockade inhibited tumor growth, we noted marked differences in the vascular morphology and function in tumors treated with NOS inhibition compared with VEGFR-2 blockade. The combination of NOS inhibition and VEGFR-2 blockade significantly increased the antivascular effects of either therapy alone. These findings suggest unique, yet complementary, effects of NOS and VEGFR-2 inhibition on the tumor vasculature.

Materials and Methods

Cell lines and culture conditions. The human pancreatic cancer cell line L3.6pl was kindly provided by I.J. Fidler, D.V.M., Ph.D. (The University of Texas M.D. Anderson Cancer Center, Houston, Texas). L3.6pl cells were cultured in minimal essential medium supplemented with 10% fetal bovine serum, penicillin-streptomycin, vitamins, sodium pyruvate, l-glutamine, and nonessential amino acids (Life Technologies, Grand Island, NY) at 37°C in 5% CO2 and 95% air. Cells were confirmed to be free of Mycoplasma.

Reagents and antibodies. The antimouse VEGFR-2 monoclonal antibody DC101 was provided by ImClone Systems, Inc. (New York, NY). The nonspecific NOS inhibitor N-nitro-l-arginine (NNLA) was purchased from Alexis Biochemicals (San Diego, CA). Antibodies for immunohistochemistry were purchased commercially: rat anti-mouse CD31/platelet-endothelial cell adhesion molecule 1 antibody from Pharmingen (San Diego, CA); peroxidase-conjugated goat anti-rat immunoglobulin G antibody from Jackson Immunoresearch Laboratories (West Grove, CA); and Alexa Fluor 594 goat anti-rat antibody from Molecular Probes (Eugene, OR).

Hoechst dye 33342 (MW 615.9) was purchased from Molecular Probes, stable 3,3-diaminobenzidine from Research Genetics (Huntsville, AL), and Gill hematoxylin solution from Sigma Chemical Co. (St. Louis, MO). Positively charged Superfrost slides were purchased from Fisher Scientific Co. (Houston, TX).

Subcutaneous implantation of tumor cells. Male athymic nude mice were purchased from the Animal Production Area of the National Cancer Institute Frederick Cancer Research and Development Center (Frederick, MD). All mice were caged in groups of five and acclimated for 2 weeks. All animal studies were conducted under guidelines approved by the Animal Care and Use Committee of M.D. Anderson Cancer Center.

L3.6pl cells were harvested from subconfluent cultures with trypsin. Trypsinization was stopped with medium containing 10% fetal bovine serum and the cells were resuspended in HBSS. Only single-cell suspensions with >90% cell viability were used for injections (as determined by trypsin blue exclusion). Mice were anesthetized by methoxyflurane inhalation (Metofane, Medical Developments, Springvale, Victoria, Australia), and under sterile conditions, 1 × 106 L3.6pl cells in HBSS (0.1 mL) were injected s.c. into the right flank of each mouse.

Therapy. Treatment was initiated 6 days after tumor cells were injected. Mice were randomly assigned to one of the following groups of 10 mice each: (a) twice weekly treatment (Monday and Thursday) with PBS by i.p. injection (control group); (b) twice weekly treatment (Monday and Thursday) with 800 µg of DC101 by i.p. injection (24); (c) 1 mg/mL NNLA in the drinking water and twice weekly i.p. injections of PBS (25, 26); or (d) combination therapy with DC101 and NNLA as described above. Tumor size was determined twice weekly using calipers. The tumor volume was calculated using the following formula: tumor volume = ab2 / 2, in which a is the longest diameter and b is the shortest diameter of the tumor.

Mice with s.c. tumors were killed on day 20, when the tumors in the control group were ∼1 cm in diameter. Five minutes before death, Hoechst 33342 (1.2 mg/mouse) was injected into the tail vein of the mice to determine tumor vessel perfusion. Tumors were excised and weighed and tumor diameters were measured with calipers. Tumor tissue was placed either in 10% buffered formalin for paraffin fixation or in optimal cutting temperature compound (Miles, Elkhart, IN) followed by freezing in liquid nitrogen for frozen tissue sections. Histology was done on s.c. tumors.

Orthotopic implantation of pancreatic cancer cells. The results of our initial study using a s.c. tumor model were validated with an orthotopic pancreatic cancer experiment. L3.6pl cells (5 × 106) were injected directly into the tail of the pancreas through a left flank incision as previously described (27). Treatment groups and protocols were similar to those described above for the s.c. implanted tumors. Mice with orthotopic tumors were killed on day 21 when mice in the control group showed signs of lethargy. Tissues were harvested for volume and weight measurements.

Immunostaining. Five representative frozen tumors from each group were analyzed for vessel count, vessel area, average vessel diameter and length, as well as for vascular perfusion. Sections 8 to 10 µm thick were stained for CD31 as previously described (28). Terminal deoxynucleotidyl transferase–mediated nick end labeling was done using a commercially available apoptosis detection kit (Promega, Madison, WI).

Image analysis. Sections stained with peroxidase-conjugated antibodies were examined using a Zeiss photomicroscope (Carl Zeiss, Thornwood, NY) equipped with a three-chip charge-coupled color camera (DXC-960 MD, Sony, Tokyo, Japan). Immunofluorescent stained slides as well as visualization of Hoescht 33342 was done using an epifluorescence microscope equipped with narrow bandpass excitation filters (Chroma Technology Corp., Brattleboro, VT), and images were captured with a C5810 Hamamatsu camera (Hamamatsu Photonics K.K., Bridgewater, NJ). All images were analyzed using Optimas image analysis software version 5.2 (Bothell, WA) and further processed with Adobe Photoshop software (Adobe Systems, Mountain View, CA). Scion software based on the NIH image program for Macintosh (Scion Corporation, Frederick, MD) was used to assess vessel area, diameter, length, and tumor perfusion. Vessel area was calculated as the number of image pixels stained positive with CD31 per high-power field. Vessel diameter and length were calculated by measuring each vessel in pixels at its largest diameter or length/image and converting pixels to micrometers. Tumor perfusion was calculated similar to vessel area as the number of image pixels for Hoescht 33342 visualization per high-power field. For each tumor section, four random high-power images (∼10 magnification) were analyzed in each quadrant of the section ∼2 mm inside the tumor-normal tissue interface.

Statistical analysis. The significance of differences among the means of the treatment groups was examined with InStat statistical software (GraphPad Software, San Diego, CA) using the Mann–Whitney U test, which were then removed from the analysis. P ≤ 0.05 was considered statistically significant.

Results

Effect of DC101 and NNLA on tumor growth. Overall, the therapies were well tolerated by the mice. Compared with the control mice, a 3% to 13% weight decrease was noted in the treatment groups with the greatest decrease observed in the combination group. No mice in any of the treatment groups appeared moribund or ill before sacrifice and no hemorrhagic complications were observed.

All mice developed primary s.c. tumors (100% tumorigenicity). Treatment with DC101 alone or NNLA alone resulted in an ~56% reduction in the mean volume of the s.c. pancreatic tumors compared with the tumors in the control group (Fig. 1; *P* < 0.008 for both individual treatments). The combination of DC101 and NNLA had an additive inhibitory effect, with an 89% reduction in the mean tumor volume compared with control tumors (Fig. 1; *P* < 0.008). Tumor weights mirrored the changes observed for tumor volume (data not shown).

**Effect of DC101 and NNLA on growth of orthotopically implanted pancreatic cancer cells.** The results from the s.c. implanted tumors were confirmed in an orthotopic pancreatic cancer model. Treatment with DC101 alone or NNLA alone resulted in an ~52% and ~63% reduction (*P* = 0.014 and *P* = 0.001), respectively, in mean tumor volume. The combination of DC101 and NNLA led to a ~78% reduction in mean tumor volumes, which was significantly less than single-agent DC101- or NNLA-treated tumors (*P* < 0.01). Tumor weights mirrored the changes observed in tumor volume.

**Effect of DC101 and NNLA on tumor vessel count and area.** Tumors were assessed for vessel count and vessel area. Compared with the control treatment, DC101 treatment resulted in a significant decrease in both mean vessel count (41%) and mean vessel area (64%; Fig. 2; *P* < 0.03 and <0.001, respectively). In contrast, treatment with NNLA resulted in a minimal reduction in mean vessel count (16%), although the mean vessel area was decreased by 38% (*P* < 0.001), likely owing to the decrease in the number of dilated vessels rather than an overall decrease in the tumor blood vessels (Fig. 2). The tumors treated with both DC101 and NNLA had the lowest mean vessel count of all the treatment groups (Fig. 2). The combination therapy group also exhibited the lowest mean total vessel area. The decreased vessel count and area observed in the combination therapy group suggested an additive antivascular effect of VEGFR-2 blockade and NO inhibition.

**Effect of DC101 and NNLA on tumor vessel morphology.** In addition to observing changes in overall vessel count and area by anti-CD31 staining, we observed morphologic changes in the tumor vessels in the treatment groups (Fig. 3). Control tumors were highly vascular with multiple dilated vessels with patent lumens. Although DC101 treatment led to a reduction in the number of tumor blood vessels, many of the vessels exhibited patent lumens, similar to the control group. In contrast, tumors treated with NNLA had few dilated vessels, and few vessels had open lumens. The combination of DC101 and NNLA decreased the total number of vessels as well as the diameter of the vessels.

**Fig. 1.** Effect of DC101 and NNLA on tumor growth. A, L3.6pl pancreatic cancer cells were s.c. injected into the flank of nude mice and treated with DC101 and/or NNLA. DC101 and NNLA, as single agents, inhibited tumor growth by ~56% relative to control treatment. The combination of DC101 and NNLA substantially inhibited tumor growth more than either therapy alone. B, representative images from tumor samples. Columns, mean; bars, SE.

**Fig. 2.** Effect of DC101 and NNLA on vessel count and area. Images were analyzed for vessel count and area in tumor sections stained with an antibody to CD31. Relative to control tumors, DC101 significantly decreased vessel count (A1) and area (B). In contrast, NNLA decreased only the vessel area significantly without a decrease in vessel count. Tumors treated with the combination of DC101 and NNLA had the lowest vessel count and area, suggesting an additive antivascular effect. hpf, high-power field. Columns, mean; bars, SE.
Effect of DC101 and NNLA on tumor vessel diameter. To quantitate the observed morphologic differences observed with DC101 and NNLA therapy, we evaluated tumor vessel diameter in random fields at the tumor-normal tissue interface on images taken of the anti-CD31-stained tumor sections. The mean vessel diameter with DC101 treatment was not significantly different than with control treatment (Fig. 4). In contrast, NNLA treatment decreased the mean vessel diameter by 42% relative to control treatment ($P < 0.001$), and the combination therapy produced a 53% decrease in mean vessel diameter ($P < 0.001$). In contrast to the additive effect of DC101 and NNLA on vessel count and area, the effect of combination treatment on vessel diameter was similar to that observed with NNLA alone, suggesting NOS inhibition was predominantly responsible for the decrease in vessel diameter.

Effect of DC101 and NNLA on tumor vessel perfusion. To assess tumor vessel perfusion, uptake of Hoechst 33342 was visualized in the adjacent tumor cells in specimens stained for CD31 (Fig. 5A). In the DC101-treated tumors, the overall number of vessels was decreased, yet the majority of remaining vessels remained perfused (76%; Fig. 5B). In contrast, NNLA...
Effect of DC101 and NNLA on tumor cell apoptosis. A, tumors treated with the combination of DC101 and NNLA showed a significant increase in terminal deoxynucleotidyl transferase–mediated nick end labeling–positive (apoptotic) cells whereas single-agent therapy had no significant effect on apoptosis. B, representative image from each treatment group.

Fig. 6. Effect of DC101 and NNLA on tumor cell apoptosis. A, tumors treated with the combination of DC101 and NNLA showed a significant increase in terminal deoxynucleotidyl transferase–mediated nick end labeling–positive (apoptotic) cells whereas single-agent therapy had no significant effect on apoptosis. B, representative image from each treatment group.

decreased the percentage of perfused vessels by 53% compared with control treatment (Fig. 5B; \( P < 0.001 \)). Similarly, tumors treated with the combination of DC101 and NNLA had a 52% decrease in perfused vessels relative to the control group, similar to NNLA alone (Fig. 5B; \( P < 0.001 \)).

The slides were further analyzed for overall tumor perfusion. DC101 alone and NNLA alone significantly decreased mean overall tumor perfusion by 43% and 54%, respectively (Fig. 5C; \( P < 0.03 \) and 0.02, respectively). Treatment with DC101 plus NNLA resulted in a 73% decrease in mean tumor perfusion (Fig. 5C; \( P = 0.001 \)).

Effect of DC101 and NNLA on tumor cell apoptosis. Terminal deoxynucleotidyl transferase–mediated nick end labeling staining revealed a slight, statistically insignificant increase (1.4- to 1.5-fold) in cellular apoptosis in tumors treated with DC101 or NNLA alone. In contrast, a 2-fold increase in cellular apoptosis was observed with the combination of DC101 and NNLA (Fig. 6; \( P = 0.036 \)).

Discussion

A predominant feature of the abnormal tumor vasculature is the heterogeneity of the blood vessels. Tumor blood vessels are irregular, tortuous, and dilated, with poor pericyte coverage and leaky endothelial cells (5). VEGF has been implicated in generating the abnormalities in tumor blood vessels and has proved to be a successful target for anti-neoplastic therapy (1). NO, a well-known vasodilator, can be up-regulated by VEGF, primarily through endothelial NOS in endothelial cells (11). Based on this information, we formulated a hypothesis that NO inhibition would have effects on the tumor vasculature comparable to those observed with VEGFR-2 blockade.

As a result of our analysis of the morphology and function of the treated tumor blood vessels, we have modified our initial hypothesis. Our analysis suggested contrasting, yet complementary, antivascular mechanisms for NOS inhibition and VEGFR-2 blockade. The combination of VEGFR-2 blockade and NOS inhibition increased the antivascular effects of either therapy alone, leading to greater tumor growth inhibition. The additive effect suggests that VEGFR-2 blockade and NO inhibition are not redundant in their mechanism of action. VEGFR-2 blockade with DC101, a monoclonal antibody that recognizes murine VEGFR-2, led to a significant decrease in vessel count as well as a decrease in tumor blood vessel length (data not shown). The majority of the remaining vessels in the tumors treated with DC101 remained patent and functional. In contrast, nonspecific NOS inhibition with NNLA had little effect on vessel count, yet decreased the mean vessel diameter, which correlated with a loss in the number of perfused vessels. In effect, NOS inhibition decreased the function of tumor blood vessels, which we theorize may be due to vasoconstriction, thereby hindering blood flow. The morphologic and functional vascular alterations in response to VEGFR-2 and NO inhibition generated an additive decrease in tumor perfusion, which translated into greater tumor growth inhibition than with either agent alone.

VEGFR-2 blockade directed at the tumor endothelial cells should theoretically inhibit endothelial NOS activity, and this led us to hypothesize that DC101 therapy would decrease the tumor vessel diameter in treated tumors. However, we found that VEGFR-2 blockade had little effect on vessel diameter, suggesting a role for other mediators in development of the abnormal vasculature of tumors. In contrast to VEGFR-2 inhibition, inhibition of NOS significantly decreased the mean vessel diameter. These observations suggest that inhibition of VEGFR-2 signaling did not inhibit all NOS activity. The likely explanation is that tumor cell NOS may contribute to the alterations observed in tumor vascular beds because tumor cell NOS induction by VEGF cannot be inhibited by DC101, owing to the species specificity of DC101 for murine VEGFR-2. We hypothesized that the vascular changes observed as a result of NOS inhibition may be due to alterations in tumor pericyte characteristics. Based on staining patterns of desmin and NG2 (traditional pericyte markers), no differences in pericyte coverage or morphology were observed between the treatment groups (data not shown).

From our studies, it is evident that NO plays a significant role in modification of the tumor vascular bed. Similar to the recent findings of Kashiwagi et al. (29), inhibition of NOS affected the tumor vasculature dramatically decreasing vessel branching and longitudinal growth. However, in contrast to our study, these investigators found that NOS inhibition led to a significant loss of branching and longitudinal vessel growth. Unlike B16 melanoma cells that did not express inducible NOS, the L3.6pl pancreatic cancer cells used in our study do express inducible NOS, and this difference may account for the
differences observed in our respective investigations. The role of tumor derived inducible NOS on tumor vascular function is less clear and thus warrants further investigation. Finally, our study supported the well-established role of targeting the tumor vasculature in antineoplastic therapy. Our results suggest that inhibiting cooperative, yet distinct, mediators of the abnormal tumor vascular network can improve antivascular and overall antineoplastic therapy. Although we are not advocating the use of NO inhibition as anticancer therapy, we believe that understanding the mechanisms of angiogenesis and the effects of antiangiogenic therapy on the vasculature will improve our ability to develop effective antiangiogenic/anti-vascular therapy and allow for identification of predictive markers of therapeutic efficacy.

References

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