The Absence of Pericytes Does Not Increase the Sensitivity of Tumor Vasculature to Vascular Endothelial Growth Factor-A Blockade

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Abstract

Recent progress with therapies targeting endothelial cells has drawn attention also to the pericytes as potential target cells for antiangiogenic therapy. Published data suggest that pericytes might confer resistance to vascular endothelial growth factor (VEGF) withdrawal in tumors. This hypothesis has been supported by experiments using tumors with reversible transgenic expression of VEGF-A as well as by individual pharmacologically targeting VEGF and platelet-derived growth factor receptor signaling in endothelial cells and pericytes using receptor tyrosine kinase (RTK) inhibitors with different specificities. However, the RTK inhibitors applied thus far are not entirely specific to the mentioned pathways, and therefore, the effects putatively attributed to pericycle targeting might reflect other antitumor effects. Here, we have reinvestigated the putative benefits of doubly targeting endothelial cells and pericytes in the treatment of experimental tumors. For this purpose, we used two highly specific tools, the pericyte-deficient pdgfbret/ret mouse and the recently developed specific anti–VEGF-A antibody G6-31, which neutralizes both murine and human VEGF-A. We generated B16, Lewis lung carcinoma, and T241 subcutaneous tumors in both pdgfbret/ret and control mice and treated these mice with G6-31. Our results fail to show any improved effect of VEGF inhibition, as measured by tumor growth or decrease in vascular density, in pericyte-deficient tumors compared with controls. Our observations suggest that additional targeting of pericytes does not increase the antitumor effect already generated by anti-VEGF drugs. Cancer Res; 70(12); 5109–15. ©2010 AACR.

Introduction

The idea of targeting angiogenesis to treat tumors was proposed almost 40 years ago (1), and since then, several approaches to block or disrupt tumor angiogenesis have been explored (2). Vascular endothelial growth factor (VEGF)-A is probably the principal growth factor in vascular development, and thus, it is a logical starting point as a target for exploring antiangiogenic therapy. In experimental models, pharmacologic targeting of the VEGF signaling pathway results in tumor growth inhibition (reviewed in ref. 3). Recently, a combination of conventional chemotherapy and a humanized anti-VEGF monoclonal antibody (bevacizumab) has been shown to increase the survival of patients with metastatic colon cancer (4) or non–small cell lung cancer (5).

Despite the promising results obtained using VEGF inhibitors, and as it occurs with other therapeutic approaches, tumors become resistant to the antiangiogenic drugs (6–8). Mechanisms that could trigger resistance to anti-VEGF therapies in tumors include genetic instability in endothelial cells (9), replacement of VEGF-A by other proangiogenic molecules such as fibroblast growth factors (10), or selective recruitment of CD11b+Gr1+ myeloid cells to refractory tumors (11). In addition, because of their involvement in blood vessel stabilization (12, 13), pericytes could promote the survival of tumor blood vessels receiving anti-VEGF drugs. This hypothesis was supported by the observation that suppression of VEGF-A expression resulted in selective pruning of tumor vessels with poor pericycle investment (14). Moreover, the remaining tumor vessels increased their pericycle coverage (14, 15).

As in developmental angiogenesis, tumor pericytes are recruited to blood vessels by platelet-derived growth factor (PDGF)-B signaling to its receptor, PDGFR-β (16). This provides a rationale for testing the effects of combined inhibition of VEGF signaling in the endothelium and PDGFR-β signaling in the mural cells. Indeed, such combined therapy resulted in enhanced tumor sensitivity compared with VEGF inhibition alone in a variety of experimental models (17, 18). However, due to the mechanism of action of the receptor tyrosine kinase inhibitors used (19), the targets might have been not only the intended VEGF receptor and PDGFR pathways but also other essential pathways that could interfere with either tumor or stromal cell (i.e., fibroblast) viability.
or function. Thus, the fundamental question about whether targeting of pericytes enhances the antitumor effect elicited by anti–VEGF-A therapies remains unanswered.

We have combined two highly specific tools to inhibit both pericyte recruitment and the VEGF-A signaling pathway. We have generated Lewis lung carcinoma (LLC), T241 fibrosarcoma, and B16 melanoma tumors in the well-established genetic model of pericyte deficiency, the pdgfbret/ret mouse (16, 20), and in littermate controls. To achieve selective inhibition of VEGF-A signaling, we have treated the tumors with the recently developed anti–VEGF-A antibody G6-31, which binds to both human and murine VEGF (21, 22). We report that the withdrawal of VEGF-A caused a delay in tumor growth in both pericyte-deficient and littermate control animals. However, both pericyte-depleted and control tumors presented the same sensitivity to treatment with G6-31 antibody as measured by delay in tumor growth. This suggests that there is no added benefit of targeting both pericytes and endothelial cells. Furthermore, VEGF-A withdrawal did not result in increased regression of tumor vasculature in the pdgfbret/ret mice compared with littermate controls. Our findings lead us to conclude that the absence of pericytes does not influence the antitumor effect already elicited by selective VEGF-A signaling pathway inhibition.

Materials and Methods

Animals

Tumors were studied in transgenic mice lacking the PDGF-B retention motif (the pdgfbret/ret mice; ref. 20). Animals were backcrossed at least seven generations against C57Bl6-J and identified by PCR genotyping of tail DNA (16). All procedures were carried out in accordance with institutional policies following approval from the animal ethical board of Northern Stockholm.

Anti–VEGF-A antibody

Anti–VEGF-A antibody, G6-31, which binds to both human and murine VEGF (21, 22), was obtained from Genentech, Inc.

Tumor experiments

T241 fibrosarcoma cells, B16 melanoma cells, and LLC cells (all cell lines syngeneic of C57Bl6 mice) were cultured in DMEM supplemented with 10% fetal bovine serum, 50 units/ml penicillin, and 0.05 μg/ml streptomycin (all reagents supplied by Life Technologies/Invitrogen). On the day of tumor induction, cells were suspended in DMEM and 1 × 10⁶ cells/100 μL were injected s.c. in the dorsal skinfold of 8-week-old mice under isoflurane gas anesthesia. We started antibody administration either 7 days (T241 and LLC models) or 10 days (B16 model) after tumor inoculation (prevention study). In a second set of experiments, tumors (LLC and T241 models) were allowed to grow up to a volume of ~0.5 cm³ before administering the antibody (intervention study).

Tumor lengths and widths were measured using a caliper, and the tumor volume (cm³) was calculated as [(width²) x (length)]/2, where width refers to the shortest measure. Because ethical regulations enforced end point of animals harboring tumors larger than 1 cm³, we sacrificed all pdgfbret/ret mice inoculated with B16 tumors that received isotype-matched control antibody after the second monitoring day (day 13 after injection).

On the first administration day, pdgfbret/ret and littermate control animals were blindly randomized to receive either 5 mg/kg i.p. of G6-31 antibody or the same dose of mouse IgG2A isotype control antibody (R&D Systems). The treatment was administered for a week, up to three doses in total. One day after the last antibody injection, the animals were sacrificed with CO₂ asphyxiation followed by cervical dislocation, and the tumors were surgically removed and fixed overnight in 4% paraformaldehyde in PBS (pH 7.4) at 4°C.

Immunohistologic methods

Tissues were cryoprotected in 30% sucrose in PBS, embedded in OCT compound (Sakura), frozen in dry ice, and stored at −80°C until sectioning. Sections (12–14 μm) were permeabilized with 0.5% Triton X-100 and 1% bovine serum albumin in PBS and then incubated with a primary antibody against CD31 (PECAM-1, 1:250; BD Pharmingen) and a FITC-conjugated anti–α-smooth muscle actin (α-SMA; 1:100; Abcam). Sections were finally incubated with Alexa Fluor dye–conjugated antibody (1:200; Molecular Probes/Invitrogen). All steps were performed in 0.125% to 0.5% Triton X-100 in PBS and finalized with an addition of 1:1,000 4′,6-diamidino-2-phenylindole (Hoechst). The sections were mounted with Mowiol 4-88 (Hoechst) supplemented with 2.5% antibleaching agent 1,4-diazabicyclo[2.2.2]octane (DABCO; Sigma). Z-stack images were obtained by confocal microscopy using a Zeiss 510 Meta microscope.

Quantification of vessel density and diameter

Vessel density was quantified using 10× images of frozen tumor sections stained with CD31 (PECAM-1). Adobe Photoshop software was used for analysis of the images, and CD31-positive vessels per field were counted in four animals per condition for the LLC tumors, seven animals per condition for the T241 tumors, and five animals per condition for the B16 tumors. For all conditions, two different images per animal were analyzed.

To quantify vessel diameter, 20× images of frozen tumor sections stained with CD31 (PECAM-1) were analyzed using the SPOT advanced image analysis software (Diagnostic Instruments, Inc.). CD31-positive vessels were localized, and the longer diameter of the vessel wall was consistently measured using the calibrated ruler provided by the software. Four animals per condition were analyzed for the LLC tumors, four animals per condition for the T241 tumors, and five animals per condition for the B16 tumors. All vessels per field were measured, but due to differences in vessel density, the number of vessels analyzed per animal was variable.

Quantification of mural cell density and vessel coverage

Mural cell density was measured by counting the amount of voxels with α-SMA–positive staining over voxels with

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CD31-positive staining using Volocity imaging software (Improvision). Voxel counts from an area of the same image showing no staining were considered as the threshold and deducted from the voxel count. Pericyte coverage was measured as percentage of α-SMA and CD31 voxel colocalization by using the “colocalization” function in the Volocity imaging software. Images used were immunostainings of frozen tumors imaged in confocal Z-stacks (63x), and a total of four tumors per condition with eight images per tumor were analyzed.

Cell toxicity assay

The sensitivity of tumor cells to the anti-VEGF antibody was assayed in vitro by mitochondrial reduction of MTT (Sigma) to formazan, as described elsewhere (23).

Statistical analysis

Data are presented as means ± SE. For comparisons of two means, two-sided, unpaired Student’s t test was used. For multiple mean comparisons, ANOVA followed by the Bonferroni post hoc test was used. Statistical analyses were carried out using GraphPad Prism v5.01 (GraphPad Software). In both cases, differences were considered statistically significant at P < 0.05.

Results and Discussion

We generated LLC, T241, and B16 tumors s.c. in both pdgfbret/ret and littermate control mice and recorded the growth kinetics of tumors receiving either G6-31 or isotype-matched control antibody. Before tumor inoculation, we used the MTT assay to establish that the exposure to G6-31 antibody did not affect the viability of these tumor cells in vitro (data not shown).

It is well established that different tumor models have different sensitivity to therapies based on VEGF-A withdrawal (11, 24). In our study, the exposure of LLC, T241, and B16 tumors to VEGF-A inhibition had distinctive antitumoral effects on each of the tumor models analyzed, as determined by tumor growth curves (Fig. 1A–C). In control animals, T241 and B16 tumors showed a delay in growth as a response to the G6-31 treatment when compared with the same models receiving control antibody (Fig. 1B and C). We recorded a negligible response to G6-31 in the LLC model when compared with tumors receiving isotype-matched control antibody (Fig. 1A). This is in line with previous observations showing that LLC tumors are refractory to VEGF-A signaling blockade (11).

LLC and B16 tumors treated with control antibody grew significantly faster in the pericyte-deficient animals compared with littermate controls (Fig. 1A and C). Previous work in the LLC model had also shown enhanced tumor growth on PDGFR-β signaling inhibition (25). The mechanism behind the increased tumor growth remains unclear and is currently under investigation.

Remarkably, the three different tumor models grown in pdgfbret/ret mice exposed to G6-31 for 7 days displayed indistinguishable final tumor volume as G6-31–treated tumors in control animals (Fig. 1A–C); that is, exposure of pericyte-deficient tumors to VEGF-A signaling inhibition did not result in further decrease of the tumor size compared with controls. The same results were obtained when T241 and LLC tumors were allowed to grow up to a volume of ~0.5 cm³ before G6-31 antibody was administered (Supplementary Fig. S1). Together, these data indicate that pericyte deficiency does not influence the sensitivity of tumors to VEGF-A withdrawal in the experimental tumor models analyzed.

Next, we sought to confirm that tumors grown in pdgfbret/ret mice are indeed deficient in recruitment of pericytes. We studied mural cell density and attachment to endothelial cells in all conditions and models by α-SMA and CD31 immunohistochemistry (Figs. 1D–I and 2) and by NG-2 and CD31 (data not shown), obtaining similar results. In control tumors treated with isotype-matched control antibody, the abundance of pericytes relative to endothelial cells was ~45% for the LLC model (Figs. 1G and 2), whereas pericytes in both T241 and B16 tumors were more abundant (~60%; Figs. 1H and I and 2). When analyzing pericyte attachment, ~35% of pericytes colocalized with endothelial cells in the LLC tumors (Figs. 1D and 2), and ~20% of pericytes colocalized with endothelial cells in both T241 and B16 tumors (Figs. 1E and F and 2). Treatment of control tumors with G6-31 antibody for 7 days barely changed either parameter in both LLC and T241 tumors (Figs. 1G and H and 2). However, B16 tumors displayed a significant increase in colocalization of CD31 and α-SMA (**, P < 0.005; Fig. 1F), indicating tighter pericyte coverage of the tumor blood vessels following VEGF inhibition (Fig. 2).

In sharp contrast, tumors grown in pdgfbret/ret mice displayed a significant reduction in both pericyte abundance and endothelial cell coverage compared with tumors generated in littermate controls (Fig. 1D–I; data not shown). Most of the blood vessels were almost depleted of pericytes (Fig. 2), and remaining pericytes were generally poorly attached to the endothelium. Treatment of these pericyte-deficient tumors with G6-31 antibody for 7 days slightly decreased both pericyte abundance and attachment to endothelial cells (Figs. 1D–I and 2), again with the exception of the B16 tumors (Figs. 11 and 2).

We conclude that, compared with controls, tumors grown in pdgfbret/ret mice have significantly fewer pericytes and that the few pericytes present do not establish proper contact with endothelial cells. Upon treatment of tumors grown in pericyte-deficient mice with VEGF-A signaling blockade for 7 days, both parameters remain largely unchanged. Therefore, pericyte abundance or their close attachment to endothelial cells does not seem to have a major influence on the sensitivity of the tumor to anti-VEGF therapies, as measured by tumor growth kinetics (Fig. 1A–C). In addition, our results from the B16 and LLC tumor models grown on pdgfbret/ret mice expand on previous observations obtained using the T241 tumor model and with NG-2 as pericyte marker (16), emphasizing the high specificity and reproducibility of our genetic model for tumor pericyte deficiency.

To further analyze the vascular effects of VEGF-A signaling blockade in tumors from both control and pdgfbret/ret mice, we determined the vessel diameter and density in all tumors and conditions. During development, absence of pericytes...
results in dilated vasculature (26). Accordingly, our analysis of vasculature from T241 and B16 tumors grown in pdgfbret/ret mice showed an increase in vessel diameter compared with controls (Fig. 3A); however, the vascular morphology of LLC tumors was unchanged (Fig. 3A). Vascular density was reduced in pericyte-deficient tumors compared with controls, in agreement with previous reports suggesting decreased angiogenesis on PDGFR-β inhibition (27).

The effect of VEGF-A withdrawal on tumor blood vessel diameter has proven to be inconsistent in different reports (15, 18), indicating possible tumor model heterogeneity. Exposure of LLC and B16 tumors grown on both control and pdgfbret/ret animals to G6-31 antibody for 7 days caused an increase in vessel diameter, which was not observed in control-treated tumors (Fig. 3A). However, G6-31 treatment on T241 tumors did not affect vessel diameter (Fig. 3A).

Tumor vascular density is a better readout of the therapeutic effect elicited by anti-VEGF-A drugs because this parameter is consistently reduced in tumors exposed to such treatment. Indeed, we recorded a reduction in tumor vessel density in all models treated with G6-31 antibody, regardless of whether they were grown in control or pdgfbret/ret animals (Fig. 3B). Because of the proposed role for pericytes in the protection of tumor vasculature on VEGF-A withdrawal (14), we
Figure 2. Immunohistochemical stainings of endothelium (CD31; red) and pericytes/vascular smooth muscle cells (α-SMA; green) in LLC (A–H), T241 (I–P), and B16 (Q–X) tumors grown in control and pdgfbret/ret animals. Mice were treated with isotype-matched control antibody or G6-31, as indicated in the figure. Left, 20× (scale bar, 50 μm); right, 63× (scale bar, 20 μm).
expected to find a greater reduction in vascular density in tumors grown in \( \text{pdgfb}^{\text{ret/ret}} \) mice. However, the reduction did not correlate with pericyte coverage or attachment to the vasculature in tumor blood vessels. The anti-VEGF-A drug caused a steeper reduction in vessel density in T241 and B16 tumors grown in control animals (~45% vessel reduction) compared with tumors grown in pericyte-deficient mice (~30%; Fig. 3B). Our results indicate that pericyte-deficient tumors do not show increased sensitivity to blockade of VEGF-A signaling, as measured by regression of tumor blood vessels.

In the LLC tumor model, we observed an interesting effect of uncoupling between angiogenesis and tumor growth. Vessel density in LLC tumors grown on \( \text{pdgfb}^{\text{ret/ret}} \) mice that received G6-31 antibody was reduced by ~65% (Fig. 3B) compared with control tumors exposed to G6-31 antibody (control animals, 149 ± 1; pericyte-deficient animals, 54 ± 6; \( P < 0.001 \)). Such dramatic reduction was not accompanied with a decrease in tumor growth because both experimental groups presented the same tumor volume (Fig. 1A). Clearly, the LLC model can tolerate a severe decrease in tumor vessel density without translating it into a measurable antitumor effect.

In summary, our data from three different experimental models indicate that the absence of pericytes does not enhance tumor sensitivity to withdrawal of VEGF-A. Moreover, tumor vasculature devoid of pericytes was resilient to regression induced by G6-31. Vessel diameter, vascular density, and pericyte coverage were characteristic of every model tested; however, all tumors were grown s.c. Because of the vascular heterogeneity in different organ sites, it might be possible that VEGF-A withdrawal affects tumor vasculature from various organs in different ways. It is also possible that the absence of pericytes renders peripheral tissues more sensitive to metastasis. This hypothesis is worth exploring, given that pericyte deficiency has already been linked to increased metastatic events in experimental mouse models (28).

Combined anti-VEGF and anti-PDGFB pathway inhibition has also been tested clinically. In a trial against human clear cell renal carcinoma, combined therapy did not improve the therapeutic effect of inhibiting VEGF alone; in addition, the combined treatment was proven to be toxic (29). Moreover, an intriguing correlation was recently found between poor pericyte vessel coverage in primary colorectal cancer and increased metastatic events in human tumors (30). VEGF-A inhibition has been reported to result in increased tumor dissemination in certain experimental conditions (31), and as already mentioned, pericyte deficiency has also been correlated with increased tumor dissemination in mouse tumor models (28). Further preclinical studies are thus granted to elucidate possible deleterious effects on PDGF or VEGF signaling inhibition.

The role of pericytes in tumor blood vessels is far from being elucidated. Recent studies indicate a possible role for RGS5, which has been found to be expressed by tumor pericytes (32, 33), in mediating immune cell transmigration into the tumor parenchyma (34). Tumor pericytes might not influence the outcome of therapeutic approaches based on VEGF-A inhibition, but further investigations will be needed to clarify potential roles for pericytes in tumor therapy.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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