Effect of Inhibition of Vascular Endothelial Growth Factor Signaling on Distribution of Extravasated Antibodies in Tumors

Tsutomu Nakahara,¹ Scott M. Norberg,¹ David R. Shalinsky,² Dana D. Hu-Lowe,² and Donald M. McDonald³

¹Cardiovascular Research Institute, Comprehensive Cancer Center, and Department of Anatomy, University of California, San Francisco and ²Department of Research Pharmacology, Pfizer Global Research and Development, San Diego, California

Abstract

Antibodies and other macromolecular therapeutics can gain access to tumor cells via leaky tumor vessels. Inhibition of vascular endothelial growth factor (VEGF) signaling can reduce the vascularity of tumors and leakiness of surviving vessels, but little is known about how these changes affect the distribution of antibodies within tumors. We addressed this issue by examining the distribution of extravasated antibodies in islet cell tumors of RIP-Tag2 transgenic mice and implanted Lewis lung carcinomas using fluorescence and confocal microscopic imaging. Extravasated nonspecific immunoglobulin G (IgG) and antibodies to fibrin or E-cadherin accumulated in irregular patchy regions of stroma. Fibrin also accumulated in these regions. Anti-E-cadherin antibody, which targets epitopes on tumor cells of RIP-Tag2 adenomas, was the only antibody to achieve detectable levels within tumor cell clusters at 6 hours after i.v. injection. Treatment for 7 days with AG-013736, a potent inhibitor of VEGF signaling, reduced the tumor vascularity by 86%. The overall area density of extravasated IgG/antibodies decreased after treatment but the change was less than the reduction in vascularity and actually increased when expressed per surviving tumor vessel. Accumulation of anti-E-cadherin antibody in tumor cell clusters was similarly affected. The patchy pattern of antibodies in stroma after treatment qualitatively resembled untreated tumors and surprisingly coincided with sleeves of basement membrane left behind after pruning of tumor vessels. Together, the findings suggest that antibody transport increases from surviving tumor behind after pruning of tumor vessels. Together, the findings suggest that antibody transport increases from surviving tumor vessel leakiness coupled with impaired fluid clearance due to the absence of functional intratumoral lymphatics and possibly contractile elements in stroma (10, 13). Binding to targets also slows antibody transport in tumors (14, 15). Nonuniformity of tumor vessel leakiness (16–18) imparts additional heterogeneity of antibody access to tumor cells (13).

Agents that block vascular endothelial growth factor (VEGF) can prune tumor vessels and reduce vascularity (19–23). Inhibition of VEGF signaling can also decrease interstitial fluid pressure in tumors (21, 22). Tumor vessels that survive treatment with VEGF inhibitors are structurally and functionally “normalized” and may deliver drugs more efficiently to tumors despite the diminished vascularity (24, 25).

Effects of angiogenesis inhibitors on tumor vessel leakiness and the distribution of extravasated antibodies in tumors are only beginning to be understood. Pruning of abnormal vasculature would be expected to reduce leakage by decreasing vascular surface area. Normalization of the vessel wall should further decrease leakiness by rectifying abnormal barrier function (25, 26). Yet, despite corrections in endothelial barrier function, tumor vessel normalization may increase the driving force for extravasation through reduced luminal resistance and interstitial pressure. Considering the multiplicity of factors, the net effect on delivery of antibodies to tumor cells is unclear. A better understanding of how angiogenesis inhibitors affect the distribution of antibodies in tumors is needed to address these issues.

The present study sought to determine, by fluorescence and confocal microscopic imaging, the distribution of extravasated antibodies in tumors and how this is affected by vascular pruning induced by inhibition of VEGF signaling. In approaching the problem, we recognized that detection of antibodies in tumors would be influenced by local concentration, which in turn would be influenced by the amount of extravasation, number and distribution of intratumoral binding sites, barriers, and other factors that govern the distribution of extravasated macromolecules (13–15, 27). Considering all these diverse properties, we selected multiple tracers to assess different factors that influence the accumulation and distribution of antibodies in tumors.

AG-013736, an inhibitor of VEGF and related tyrosine kinases, was used to inhibit VEGF signaling (23, 28, 29) in two well-characterized tumor models, spontaneous pancreatic islet tumors in RIP-Tag2 transgenic mice (30) and implanted Lewis lung carcinomas. Fluorescent microspheres were injected into the bloodstream to pinpoint leakage from tumor vessels (31), with recognition that their large size would limit movement away from sites of extravasation. Nonspecific immunoglobulin G (IgG) was injected as a surrogate...
for antibodies having little or no specific binding to identify preferential pathways and barriers to antibody movement in tumors. Distribution of fibrin was used as an indicator of where extravasated macromolecules do or do not accumulate in tumors (18) and antifibrin antibody was injected to determine the accessibility of these sites to macromolecular probes. Extravasated IgG and antibodies were readily detected in patchy regions of stroma where fibrin accumulated but not next to tumor cells. We therefore asked whether antibodies that target epitopes on tumor cells can achieve a detectable local concentration within tumor cell clusters where nontargeted antibodies do not. This question was addressed by injecting an antibody to E-cadherin on tumor cells. Together, the results showed that VEGF inhibition pruned 86% of tumor vessels but did not qualitatively change the distribution of extravasated antibodies. Of all those tested, only the antibody to E-cadherin was detected next to tumor cells, and this was maintained after tumor vessel pruning.

Materials and Methods

Tumor models and treatment. Spontaneous pancreatic islet cell tumors were studied in 10- to 11-week-old RIP-Tag2 transgenic mice with a C57BL/6 background (30). Transgene-positive mice were identified by genotyping tail-tip DNA by PCR. Complementary experiments were done on 7- to 8-week-old wild-type C57BL/6 mice implanted under the dorsal skin with a 1-mm3 piece of Lewis lung carcinoma that was allowed to grow for 9 days before treatment (23). Mice were housed under barrier conditions in the animal care facility at the University of California, San Francisco. Mice were treated by i.p. injection of AG-013736 (25 mg/kg in a volume of 5 mL/kg) or its vehicle (5 mL/kg; 3 parts polyethylene glycol 400 to 7 parts acidified H2O, pH 2-3) twice daily for 7 days (29). Selection of this agent and dose were based on results of previous studies involving multiple agents, doses, and durations of treatment (23, 32–34). AG-013736 reduces the vascularity of RIP-Tag2 tumors by ~80% and Lewis lung carcinoma tumors by ~50% (23). All experimental procedures were reviewed and approved by the University of California-San Francisco Institutional Animal Care and Use Committee.

Injection of tracers and fixation by vascular perfusion. At the end of the treatment period, mice were anesthetized with ketamine (87 mg/kg) plus xylazine (13 mg/kg) plus atropine (0.1 mg/kg) injected i.m., and then IgG, antibodies, or microspheres were injected i.v. to identify leakage sites and to determine the distribution of extravasated tracers in tumors. The tracers were (a) fluorescent 50-nm polymer microspheres (250 μL/kg; R50, Duke Scientific, Palo Alto, CA); (b) rat or goat nonspecific IgG (2.5 mg/kg; Jackson ImmunoResearch, West Grove, PA); (c) rabbit polyclonal antifibrinogen/fibrin antibody (2.5 mg/kg; DAKO, Carpinteria, CA); or (d) goat polyclonal anti-E-cadherin antibody (2.5 mg/kg; R&D Systems, Minneapolis, MN). In pilot studies, we also used tetramethylrhodamine isothiocyanate-dextran (500 mg/kg; average molecular weight 155,000, T1287, Sigma, St. Louis, MO) and Cy3-conjugated rat IgG (2.5 mg/kg; 012-160-008, Jackson ImmunoResearch).3

Tracers were diluted in 0.9% NaCl (Vector Laboratories, Burlingame, CA) to a volume of 100 to 200 μL, injected via a tail vein, and allowed to circulate for 6 hours before the tissues were fixed by vascular perfusion. This time point was based on data from a previous study showing that accumulation of circulating antibodies or nonspecific IgG in RIP-Tag2 tumors peaks at 6 hours and then plateaus or decreases (18). In some mice, FITC-labeled Lycopersicon esculentum lectin (100 μg in 100 μL of 0.9% NaCl; Vector Laboratories) was injected i.v. at 3 minutes before perfusion of fixative to label the functional vasculature (23). The chest was opened rapidly and the vasculature was perfused for 5 minutes at a pressure of 120 mm Hg with fixative (1% paraformaldehyde in PBS, pH 7.4) from an 18-gauge cannula inserted into the aorta via an incision in the left ventricle. The right atrium was incised to create a route for the fixative to exit. After the perfusion, tissues were removed and stored in fixative for 1 hour at 4°C. Specimens were rinsed several times with PBS and infiltrated overnight with 30% sucrose in PBS at 4°C, embedded in Optimal Cutting Temperature (OCT) compound (Sakura Finetek, Torrance, CA), and frozen at ~80°C.

Injected tracer identification and immunohistochemistry. Injected microspheres were identified in cryostat sections by their intrinsic fluorescence. Injected antibodies or IgG were identified in cryostat sections by staining with fluorescent species-specific secondary antibodies. Tissues sections were generally also stained by conventional on-section immunochemistry to localize tumor components, except when the injected tracer was followed by FITC-lectin staining of the vasculature.

Tissue sections were cut with a cryostat at a thickness of 80 μm and dried on Super Frost Plus slides (Fisher Scientific, Santa Clara, CA). Sections on slides were fixed in 3% (v/v) ethanol containing 0.1% (w/v) diaminobenzidine for 10 minutes, then washed 3 times with PBS. Slides were incubated for 24 hours at room temperature with one or more primary antibodies, diluted in 5% normal serum in PBS, 0.1% Triton X-100, to label the functional vasculature (23). The chest was opened rapidly and the vasculature was perfused for 5 minutes at a pressure of 120 mm Hg with fixative (1% paraformaldehyde in PBS, pH 7.4) from an 18-gauge cannula inserted into the aorta via an incision in the left ventricle. The right atrium was incised to create a route for the fixative to exit. After the perfusion, tissues were removed and stored in fixative for 1 hour at 4°C. Specimens were rinsed several times with PBS and infiltrated overnight with 30% sucrose in PBS at 4°C, embedded in Optimal Cutting Temperature (OCT) compound (Sakura Finetek, Torrance, CA), and frozen at ~80°C.

Fluorescence imaging. Specimens were examined with a Zeiss Axiohot fluorescence microscope equipped with single, dual, and triple fluorescence filters. Digital fluorescence microscopic images were captured with a low-light, externally cooled, three-chip charge-coupled device camera (480 × 640 pixel RGB-color images, CoolCam, SciMeasure Analytical Systems, Atlanta, GA). Tissue sections were counterstained with Ponceau S (0.1% × 120 μM for RIP-Tag2 tumors (10× objective, 1× Optovar) and 1920 × 2560 μM for Lewis lung carcinoma tumors (5× objective, 1× Optovar). Measurements of area density and fluorescence intensity were made on these images. Images for colocalization measurements were obtained with a Zeiss LSM 510 confocal microscope with argon, helium-neon, and UV lasers (512 × 512 pixel RGB-color images).

www.aacrjournals.org 1435 Cancer Res 2006; 66: (3). February 1, 2006
Measurement of tracer distribution and tumor vascularity. An index of area density (proportion of sectional area) was measured to quantify the abundance of patent tumor vessels (lectin staining of perfused tumor vessels), tumor vascularity (CD31-positive tumor vessels), type IV collagen, fibrin, and extent of distribution of injected tracers in RIP-Tag2 tumors and Lewis lung carcinoma tumors (23). Digital fluorescence microscopic images of tracers, blood vessels, and other tumor components in 80-μm-thick cryostat sections were analyzed using ImageJ. Images were captured from sections of four to five tumors, ranging in diameter from 0.5 to 1.0 mm, in each RIP-Tag2 mouse and three to five regions of Lewis lung carcinoma tumors. Each group consisted of three to four mice. Nontumor tissues were excluded from measurements by using the outlining function of ImageJ.

Specific staining of extravasated tracers or tumor components was distinguished from background by applying threshold values of fluorescence intensity (range, 0-255) that gave the greatest signal-to-noise ratio for measuring specific immunoreactivity (23). Area densities of specific immunoreactivity were calculated as the proportion of pixels having a fluorescence intensity value ≥ 20 for tracers in RIP-Tag2 tumors, 10 to 20 for lectin staining in RIP-Tag2 tumors, and 50 for tracers in Lewis lung carcinoma tumors (23). Area densities expressed per tumor vessel were calculated as the ratio of area density of extravasated tracer to area density of CD31 immunoreactivity or lectin staining. Mean values were calculated for all tumors or tumor regions in each mouse, and from these values overall means were calculated for each group (three to four mice per group).

Mean fluorescence intensity measurements. Mean fluorescence intensity of extravasated nonspecific IgG was measured as an index of local concentration in fluorescence microscopic images. Analysis was done on 80-μm sections of RIP-Tag2 tumors prepared 6 hours after injection of nonspecific IgG or, as a control, injection of vehicle. Sections were stained with Cy3-labeled secondary antibody. Camera gain was calibrated on vehicle-injected sections, which had no foreground (rat IgG) fluorescence, to give barely visible background fluorescence in digital images (10× objective, 1× Optovar, Cy3 filter). In these images, 97% of pixels had a fluorescence intensity of <15 (intensity range, 0-255; ref. 23). Specific fluorescence (threshold) was thus established as fluorescence intensity ≥15. Fluorescence microscopic images were then obtained from four to five tumors in each IgG-injected mouse (three to four mice per group) with camera gain set as for the calibration sections. Camera gain was adjusted to maintain the background fluorescence at the calibration level. Nontumor tissues were excluded. RGB images of Cy3 fluorescence were converted to 8-bit gray scale images and fluorescence intensity was determined for each pixel above the threshold using ImageJ (23). Mean fluorescence intensity of each image was calculated as the overall sum of the number of pixels at each fluorescence intensity equal to or greater than the corresponding threshold (fluorescence intensity ≥15 to exclude background fluorescence), times that intensity, divided by the total number of pixels with intensity ≥15 (23). Images of tumor sections containing fluorescent microspheres without further staining were analyzed similarly.

Distribution of fibrin and E-cadherin. The distribution of fibrin was determined in sections of RIP-Tag2 tumors stained by conventional immunohistochemistry using the rabbit polyclonal antifibrin antibody or goat polyclonal antifibrin antibody. The distribution of extravasated rabbit polyclonal antibody, identified by staining with anti-rabbit secondary antibody, was compared to the distribution of fibrin, identified by conventional immunohistochemical staining of the same section with goat polyclonal antifibrin antibody. Alternatively, distributions of injected antibody and fibrin were compared in two sequential 80-μm sections, the first stained with anti-rabbit secondary antibody and the second stained by conventional on-section immunohistochemistry using the rabbit polyclonal antifibrin antibody. Only the injected antibody was stained in the first, but both the injected antibody and fibrin were stained in the second.

To determine whether AG-013736 had a detectable effect on E-cadherin expression in RIP-Tag2 tumors, 80-μm sections were stained by conventional on-section immunohistochemistry using the same anti-E-cadherin antibody that was used for i.v. injection. Area density of E-cadherin immunoreactivity was measured in tumors 0.5 to 1 mm in diameter from mice treated with AG-013736 or its vehicle for 7 days.

Colocalization of extravasated antibodies with tumor components. The distribution of fibrin, extravasated IgG, antifibrin antibody, or anti-E-cadherin antibody in tumors was assessed by determining the extent of colocalization with basement membrane (type IV collagen, nidogen) or tumor cells (insulin). Preliminary studies confirmed that both type IV collagen and nidogen immunoreactivities marked the distribution of basement membrane in RIP-Tag2 tumors, and most of the staining was associated with blood vessels (37). Extent of colocalization was determined in confocal microscopic images prepared as 15-μm-thick projections from z-axis stacks of 1-μm optical sections of immunohistochemically stained 80-μm cryostat sections of RIP-Tag2 tumors (20× objective). Confocal images of the red channel (fibrin, extravasated IgG, or antibody labeled with Cy3-conjugated secondary antibody) and green channel (type IV collagen, nidogen, or insulin labeled with FITC-conjugated secondary antibody) of the same field were captured separately and saved as pairs of digital files. Pixels that colocalized in space in red-green image pairs were identified with the Colocalization plug-in function of ImageJ. Colocalized pixels were defined as pixels having red and green channels with fluorescence intensities above their respective thresholds (5-10 for red channel; 2-10 for green channel). Amount of colocalization was calculated as the proportion of total pixels for fibrin, extravasated IgG, or antibody that colocalized with type IV collagen, nidogen, or insulin.

Statistical analysis. Values are expressed as mean ± SE. The significance of differences between pairs of mean values was determined by Student’s t test. P < 0.05 was considered significant.

Results

Extravasation of nonspecific IgG and microspheres in tumors. Sections of RIP-Tag2 tumors examined 6 hours after i.v. injection of nonspecific IgG had a distinctive patchy network of extravasated IgG identified by intense immunofluorescence (Fig. 1A). After treatment with AG-013736 for 7 days, the pattern was qualitatively similar but had fainter fluorescence (Fig. 1B). Area density measurements showed that AG-013736 reduced tumor vascularity, as reflected by lectin-stained blood vessels, by 86% (Fig. 1C), but reduced the overall extent of extravasated nonspecific IgG by 69% (Fig. 1D). Because of the larger reduction in vascularity, extravasated IgG values expressed per vessel were on average twice as high in treated tumors (Fig. 1E).

The brightness of IgG immunofluorescence in tumors decreased after treatment, consistent with a lower IgG concentration from reduced extravasation (Fig. 1F and G). This reduction was evident as decreased peak heights in fluorescence intensity surface plots (Fig. 1H) and as decreased mean intensities, which were 18% less in AG-013736-treated tumors (28.5 ± 1.5; intensity range, 0-255) than in vehicle-treated tumors (34.7 ± 1.6; P < 0.05, n = 3-4 mice per group).

The distribution of extravasated 50-nm microspheres in RIP-Tag2 tumors differed from extravasated IgG. Extravasated microspheres accumulated in focal regions (Fig. 1I) unlike the irregular patchy network of extravasated IgG. Treatment with AG-013736 reduced by 91% the extravasation of microspheres (Fig. 1J). Area density measurements showed this reduction was 37% more than the reduction in vascular density (Fig. 1K-M), consistent with decreased leakage per vessel. Surface plots of microsphere fluorescence showed a reduction in number, but not height, of peaks after treatment (Fig. 1N-P), indicating that mean fluorescence intensity did not change appreciably (vehicle, 52.8 ± 2.8 versus AG-013736, 49.3 ± 2.4; n = 4 mice per group). Little change in microsphere fluorescence intensity, despite the large decrease in microsphere leakage, was evidence that brightness was
determined more by the fixed inherent fluorescence of microspheres than by their concentration.

Confocal microscopic imaging confirmed that the irregular, patchy network of extravasated IgG lacked a consistent association with individual tumor vessels (Fig. 2A) and changed little after AG-013736 (Fig. 2B). Tumor vascularity, stained by i.v. injection of FITC-lectin, was reduced 86% after AG-013736 (C). IgG was reduced overall (D) along with vascularity but was increased per surviving tumor vessel (E). Reduction in IgG concentration in tumors after AG-013736 was reflected by decreased immunofluorescence (F and G) and by lower peaks in surface plots of fluorescence intensity (H). Focal leakage sites of fluorescent microspheres in vehicle-treated tumors (I) were sparse after AG-013736 (J). After treatment, tumor vascularity decreased (K) and microsphere extravasation showed an even greater reduction (L). When microsphere area density was scaled to tumor vascularity, values decreased even further (M). Reduction in microsphere extravasation is evident microscopically (N and O) and by fewer peaks in fluorescence intensity surface plots (P). Peak height of microspheres did not change, consistent with constant fluorescence of individual microspheres. Columns, mean; bars, SE (n = 3-4 mice per group). *, P < 0.05, different from vehicle. Bar, 200 μm.

Factors determining the distribution of extravasated macromolecules in tumors. We next sought to identify some of the factors that determine the distribution of extravasated antibodies in tumors. As a first step, we identified the distribution of two major compartments of RIP-Tag2 tumors—stroma, as reflected by blood vessels (CD31) and basement membrane (type IV collagen), and tumor cell clusters, as defined by insulin immunoreactivity of islet cells—with the goal of determining their relationship to the distribution of extravasated antibodies.

CD31-positive blood vessels were abundant around tumor cell clusters of untreated RIP-Tag2 tumors (Fig. 3A). After AG-013736,
CD31 staining was markedly reduced (Fig. 3B), consistent with the 86% reduction in lectin-stained tumor vessels (Fig. 1C). By comparison, type IV collagen staining of basement membrane was about the same under the two conditions (Fig. 3C and D). Measurements showed that type IV collagen was reduced only 11% in treated tumors (area density, 41 ± 2%) in comparison with baseline (46 ± 2%, n = 4 mice per group) as previously reported (23).

CD31 and type IV collagen immunoreactivities did not completely match in some regions of untreated tumors (Fig. 3A, C, E, and F) but the extent of mismatch was greatly increased by AG-013736 treatment (Fig. 3B, D, G, and H). Strands of type IV collagen lacking CD31 staining had the same appearance as empty sleeves of basement membrane found after regression of endothelial cells in tumors and normal organs (23, 33, 34).

Extravasated IgG preferentially accumulated in regions marked by type IV collagen immunoreactivity (Fig. 4A-C), both under baseline conditions and after treatment with AG-013736. Measurements showed similarly extensive colocalization of IgG with type IV collagen under baseline conditions (84 ± 1%) and after AG-013736 (86 ± 1%, n = 3 mice per group). Importantly, some extravasated IgG was located in regions of type IV collagen that lacked CD31 staining (Fig. 4D-F), suggesting that extravasated IgG accumulated not only in stroma around surviving tumor vessels but also in empty sleeves of basement membrane left behind by regressing tumor vessels.

**Distribution of fibrin and extravasated antifibrin antibody in tumors.** Fibrin accumulations are a common feature of tumors that provide a historical record of sites of leakage from tumor vessels (18, 38). In RIP-Tag2 tumors, the distribution of fibrin (Fig. 5A) resembled the patchy network of extravasated nonspecific IgG (Fig. 1A and F). The distribution of fibrin identified in tumors by conventional on-section immunostaining with goat antifibrin antibody (Fig. 5A) was similar, but not identical, to the distribution of labeling in the same section by extravasated rabbit antifibrin antibody (Fig. 5B and C). The labeling patterns by extravasated antibody and, in an adjacent section, by on-section immunohistochemistry using the same antibody were also similar (Fig. 5D and E). Neither fibrin nor labeling by extravasated antifibrin antibody was as extensive or as uniformly distributed as the tumor vasculature marked by CD31 staining (Fig. 5D and E). As was shown by extravasated IgG and antifibrin antibody, sites of leakage and/or accumulation of antibodies in RIP-Tag2 tumors were patchy and heterogeneously distributed.

Because of the potent pruning effect of AG-013736 on the vasculature of RIP-Tag2 tumors, we asked whether the accumulation of fibrin or extravasated antifibrin antibody was similarly reduced. We found that treatment had little effect on the area density of fibrin (Fig. 5F). After AG-013736 for 7 days, the overall area density of extravasated antifibrin antibody decreased (Fig. 5F), but the pattern of leakage changed little (Fig. 5G and H), and when scaled to the number of surviving tumor vessels, the values were greater than baseline (Fig. 5J).

Similar studies done on Lewis lung carcinoma tumors showed robust leakage of antifibrin antibody at the tumor-host interface but little was evident within tumors under baseline conditions (Fig. 5F). After treatment with AG-013736 for 7 days, CD31-positive tumor vessels decreased by 45% (vehicle, 11 ± 2%; AG-013736, 6 ± 1%, n = 3-4 mice per group). Patchy regions of extravasated antibody were scattered within Lewis lung carcinoma tumors (Fig. 5K) and the area density of extravasated antifibrin antibody expressed per surviving tumor vessel increased 4-fold (Fig. 5J).

Most fibrin in untreated RIP-Tag2 tumors was located in regions of basement membrane as marked by nidogen immunoreactivity (Fig. 6A and B). Extravasated antifibrin antibody had a similar distribution (Fig. 6C and D). Measurements showed that most fibrin (84 ± 3%) and extravasated antifibrin antibody (89 ± 2%, n = 3 mice per group) colocalized with nidogen. After treatment with AG-013736, both fibrin (Fig. 6E and F) and antifibrin antibody (Fig. 6G and H) remained closely associated with basement membrane as supported by extensive colocalization of fibrin (85 ± 3%) and extravasated antibody (84 ± 2%, n = 3 mice per group) with nidogen. Most nidogen staining was associated with empty sleeves of basement membrane left behind by pruned tumor vessels.
Distribution of extravasated anti-E-cadherin antibody in RIP-Tag2 tumors. Extravasated IgG and antifibrin antibody were readily detected in stroma around tumor cells of RIP-Tag2 tumors but not within tumor cell clusters. Because neither immunoglobulin binds to tumor cells, we asked whether extravasated antibodies that bind surface proteins on tumor cells could label tumor cell clusters. For this purpose, we used an antibody that recognizes the extracellular domain of E-cadherin, an adhesion molecule expressed on cells of RIP-Tag2 adenomas. E-cadherin is also expressed by normal pancreatic \( \beta \)-cells and other epithelial cells but not invasive RIP-Tag2 tumors (39, 40).

At 6 hours after injection, anti-E-cadherin antibody, like other antibodies tested, accumulated in a patchy network in tumor stroma; some also labeled the surface of insulin-positive tumor cells (Fig. 7A-C). Treatment with AG-013736 did not qualitatively change the distribution of extravasated anti-E-cadherin antibody; both the patchy stromal network and tumor cells were labeled (Fig. 7D-F). Area density measurements showed that AG-013736 reduced the overall distribution of extravasated anti-E-cadherin antibody by 43% but increased the amount per vessel by 152% (Fig. 7G).

Tumor cell labeling by extravasated anti-E-cadherin antibody had a dotlike appearance that colocalized with insulin immunoreactivity (Fig. 7C and F). Approximately 22% of anti-E-cadherin pixels colocalized with insulin under baseline conditions (Fig. 7H and K) and after treatment with AG-013736 (Fig. 7l and L). Most of the remaining anti-E-cadherin antibody in tumors was in the patchy stromal network. Extravasated antibody also labeled pancreatic acini that express E-cadherin (Fig. 7D). No extravasated nonspecific IgG was detected within tumor cell clusters (Fig. 7J) or colocalized with insulin (Fig. 7M).
To determine whether the overall decrease in anti-E-cadherin antibody in RIP-Tag2 tumors after AG-013736 could be explained by a reduction in E-cadherin expression, we compared the immuno-reactivity after on-section staining with or without treatment. E-cadherin was present on tumor cells in early- and mid-stage RIP-Tag2 tumors in both groups. Area density measurements showed that E-cadherin immunoreactivity was tended to be less (not significant) in mice treated with AG-013736 (49 ± 4%) compared to vehicle-treated mice (59 ± 4%; n = 3-4 mice per group).

Discussion

The present study sought to determine the effect of inhibition of VEGF signaling on the distribution of extravasated antibodies in tumors. Using fluorescence and confocal microscopic imaging to study tumors in RIP-Tag2 transgenic mice, we found that extravasated 50-nm microspheres stayed close to vessel walls but extravasated nonspecific IgG, antifibrin antibody, and anti-E-cadherin antibody accumulated in irregular patchy networks that lacked a close association with tumor vessels. Fibrin had a patchy distribution similar to that of antibodies. Anti-E-cadherin antibody, which targets epitopes on tumor cells in RIP-Tag2 adenomas, was the only antibody to accumulate in detectable amounts in tumor cell clusters. Inhibition of VEGF signaling by AG-013736 for 7 days resulted in 86% reduction in tumor vascularity. Microsphere accumulation decreased even more than the reduction in vascularity. Area density of extravasated antibodies in tumors decreased after inhibition of VEGF signaling but, when expressed per surviving tumor vessel, was less than the reduction in vascularity. Similar findings were obtained in Lewis lung carcinoma tumors. Area density of anti-E-cadherin antibody within tumor cell clusters was also reduced less by treatment than was tumor vascularity.

Figure 4. Comparison of distributions of extravasated nonspecific IgG at 6 hours after injection and vascular basement membrane in RIP-Tag2 tumors with or without VEGF inhibition. Confocal micrograph showing the patchy pattern of extravasated IgG only vaguely associated with CD31-positive blood vessels (A). Blood vessels had a similar but not identical pattern to basement membrane, marked by type IV collagen immunoreactivity (B). Most extravasated IgG coincided with regions of type IV collagen (C). After AG-013736, the distribution of IgG was still patchy and vaguely associated with blood vessels (D, arrows). Empty sleeves of basement membrane were abundant (E). Extravasated IgG still colocalized with basement membrane (F). Bar, 75 μm (A, B, D, and E); 40 μm (C and F).
vascularity. Antibodies accumulated in patchy regions of tumor stroma that, after treatment, included sleeves of basement membrane left behind by regressing tumor vessels. Together, the findings suggest that antibody transport increased from tumor vessels that survived inhibition of VEGF signaling. Basement membrane sleeves may facilitate the movement of the extravasated antibodies in these tumor models. Antibodies preferentially distribute in tumor stroma but also can reach tumor cells and accumulate there if binding sites are accessible.

Plasma leakage from tumor vessels. Leakage from tumor vessels depends on size, charge, and other properties of extravasated molecules or particles (36, 41, 42). Amount of leakage also depends on vascular surface area, endothelial permeability, driving force for extravasation, and interstitial fluid pressure (9–12). Dose, plasma kinetics, specific binding to targets, metabolism, extracellular matrix composition, and lymphatic outflow are additional factors that influence movement of extravasated antibodies in tumors (14, 15, 43). The effect of high affinity binding of antibodies to tumor cell antigens ("binding site barrier") on antibody movement in tumors has been shown by using mathematical models (44, 45).

Rather than quantifying vascular leakiness or permeability per se, we focused on factors that influence the distribution of antibodies in tumors, particularly after the tumor vasculature was pruned by inhibition of VEGF signaling. We initially used 50-nm microspheres to identify leaky sites in RIP-Tag2 tumors. Although much larger than antibodies and unlikely to detect all sites where antibodies leak, microspheres proved useful because they moved little after extravasation and labeled individual leaky vessels in a way that antibodies cannot. Leaky sites were heterogeneously distributed in RIP-Tag2 tumors, involved only a small proportion of vessels, and decreased in abundance after VEGF inhibition.
Next, we injected nonspecific IgG to identify barriers and preferential pathways for transport of immunoglobulins with little or no specific binding in tumors. We found that IgG accumulated in irregular patchy networks that did not have a close association with individual tumor vessels as previously reported (18, 46). Most patches of IgG coincided with regions of stroma that stained for basement membrane markers, type IV collagen, or nidogen. Inhibition of VEGF signaling reduced the accumulation of IgG but not as much as the reduction in vascularity. Because the distribution of immunoglobulins in tumors depends on multiple factors, the leakage pattern of nonspecific IgG was used to show the distribution of antibodies without targets in tumors.

Macromolecules extravasate through intercellular gaps, transcellular transport, and other mechanisms (5–8, 47, 48). Decreased vascular surface area from vessel pruning was probably the dominant factor responsible for the reduction in overall leakage of microspheres and antibodies after inhibition of VEGF signaling. Improvement of endothelial barrier function may have contributed to the reduction in microsphere leakage, to the extent that it decreased more than vascularity.

Despite changes that tend to reduce leakage, antibody area density expressed per vessel increased and the overall patchy pattern of accumulation was qualitatively similar after treatment. Increased driving force for extravasation, resulting from decreases in vascular resistance and interstitial fluid pressure, would partially offset decreases in endothelial surface area and permeability. Normalization of the extravascular environment through decreased leakage, changes in stromal elements, or increased fluid flux out of tumors would lower interstitial pressure (21, 22, 25, 26). Although all the consequences of actions of VEGF inhibitors on tumors are not clear, they collectively could improve the delivery of antibodies despite decreased vascularity.

**Factors determining the distribution of extravasated macromolecules in tumors.** Inhibition of VEGF signaling can lower interstitial fluid pressure, presumably by normalizing vascular permeability and architecture, and thereby improve delivery of macromolecules (21, 22, 25, 49). However, the heterogeneous, patchy distribution of extravasated IgG and antibodies in RIP-Tag2 tumors was still evident after AG-013736 despite the 86% reduction in vascularity and changes in permeability and driving force accompanying vessel normalization.

In exploring other factors responsible for the patchy distribution of extravasated antibodies, we examined the contribution of basement membrane in tumor stroma. The patchy distribution of extravasated IgG, antibodies, and fibrin roughly matched the distribution of basement membrane. In RIP-Tag2 tumors, tumor vessels are covered by loose, sometimes multilayered, basement...
membrane (37). Most extravasated particles (e.g., microspheres, colloidal carbon, and liposomes) accumulate between endothelial cells and basement membrane of leaky vessels (16, 31). Basement membrane limited the movement of extravasated microspheres but not antibodies away from vessel walls (18, 46).

Vascular basement membrane can persist after endothelial cells regress (50, 51). In RIP-Tag2 tumors, the amount of basement membrane marked by type IV collagen changes little after VEGF inhibition, but some sleeves lack endothelial cells (23). Empty sleeves of basement membrane are also present in the trachea, thyroid, and other organs where capillaries undergo regression after inhibition of VEGF signaling (32–34). Tumor cells also make basement membrane but most type IV collagen and nidogen immunoreactivity in RIP-Tag2 tumors is associated with the vasculature (37).

The preferential accumulation of extravasated antibodies in type IV collagen/nidogen rich regions left behind after blood vessels regress raises the possibility that empty basement membrane sleeves influence antibody distribution in tumors. Here, the word “empty” may be misleading because there is no evidence the sleeves have a lumen. Instead, the sleeves probably collapse after endothelial cells regress and accumulate antibodies more as wicks than as tubes. If the sleeves accumulate more antibody than does basement membrane of intact tumor vessels, the sleeves could contribute inhomogeneity to available volume fractions.

Extracellular matrix provides a path for transport of extravasated fluid and soluble molecules in tumors (52). Although clearly not a route for blood flow in our tumor models, basement membrane may facilitate extravascular fluid and solute transport, similar in some respects to vasculogenic mimicry (53). Our results raise the possibility that sleeves of vascular basement membrane provide preferential routes for distribution and accumulation of antibodies in tumors.

Figure 7. Binding of extravasated anti-E-cadherin antibody to tumor cells in RIP-Tag2 adenomas. Unlike the other antibodies used, extravasated anti-E-cadherin antibody labeled tumor cell clusters (A–C, arrows) in addition to patchy regions of stroma. Tumor cell labeling was punctate. After treatment with AG-013736 for 7 days, tumor cell labeling was reduced but the pattern was qualitatively similar to baseline conditions (D–F). Extravasated anti-E-cadherin antibody labeled some regions with few or no blood vessels (E, arrows). Measurements showed that, after AG-013736, area density of anti-E-cadherin antibody decreased overall (G, top) but increased per surviving tumor vessel (G, bottom). Colocalization of extravasated anti-E-cadherin antibody with insulin in tumor cells (H, I, K, and L) was suggestive of intracellular labeling. No colocalization of extravasated nonspecific IgG with insulin was detected (J and M). Amount of colocalization of anti-E-cadherin pixels and insulin pixels was 22% under baseline conditions and after AG-013736. Columns, mean; bars, SE (n = 4 mice per group). *, P < 0.05, different from vehicle. Bar, 100 μm (A); 25 μm (B, C, E, and F); 90 μm (D); 12.5 μm (H–M).
Distribution of fibrin deposits and extravasated antifibrin antibody in tumors. Fibrin is a common feature of tumor stroma (38, 54, 55). Fibrin is deposited in regions of tumors where fibrin monomers polymerize after cleavage of extravasated fibrinogen (38, 56, 57).

In RIP-Tag2 tumors, fibrin deposits have a patchy distribution (18) that matched the pattern of extravasated antibodies and coincided with basement membrane in stroma. The distribution of fibrin was not qualitatively affected by the potent effect of AG-013736 on blood vessels. As the amount of fibrin in tumors reflects the balance of rates of formation and fibrinolysis (55, 58), the stability of fibrin after VEGF inhibition is consistent with a slow turnover in the tumor stroma. It also indicates that the reduction in labeling by extravasated antifibrin antibody after treatment resulted from reduction in antibody extravasation rather than loss of fibrin.

The coherence of the distributions of fibrin and extravasated antifibrin antibody in RIP-Tag2 tumors was assessed by using two different antifibrin antibodies, one injected and the other used for on-section immunohistochemistry. Some extravasated antibody did not colocalize with fibrin deposits and vice versa. Regions of mismatch may reflect accumulation of unbound extravasated antifibrin antibody, historical accumulations of fibrin that were not accessible to extravasated antibody, or subtle differences in binding of the two antifibrin antibodies used for immunohistochemical staining.

Extravasation of antifibrin antibody in untreated Lewis lung carcinoma tumors, unlike RIP-Tag2 tumors, was conspicuous at the tumor-host interface but minimal in the interior of the tumor. Leakiness at the tumor-host interface is a well-documented feature of s.c. implanted tumors (16). After treatment with AG-013736, the distribution of extravasated antifibrin antibody within Lewis lung carcinoma tumors broadened despite pruning of 47% of the vasculature.

Distribution of extravasated anti-E-cadherin antibody in tumors. Neither extravasated IgG nor antifibrin antibody was detected in RIP-Tag2 tumor cell clusters. Although this could be explained by lack of accessibility to this compartment, absence of immunoreactivity does not necessarily mean absence of antibody because only amounts exceeding the threshold of detection were visible.

To increase the sensitivity of antibody detection near tumor cells, we asked whether injected antibodies that recognize surface proteins on tumor cells can reach their target in RIP-Tag2 tumors. We reasoned that binding to membrane proteins could increase the local concentration of antibody above the threshold for detection. We found that extravasated antibody to the extracellular domain of E-cadherin not only accumulated in the tumor stroma but also labeled tumor cells. Some pancreatic acini next to tumors were also stained by extravasated antibody from tumor vessels. Some pancreatic acini next to tumors were also stained by extravasated antibody from tumor vessels. Tumor cell labeling had a dotlike appearance at 6 hours after injection of the antibody. As E-cadherin is removed from the basement membrane by endocytosis (59), the dotlike staining may represent internalization of antibody bound to E-cadherin into tumor cells. Successful targeting of E-cadherin antibody to tumor cells indicates that both IgG and antifibrin antibody probably had access to the compartment but did not achieve detectable concentrations.

Detection of tumor vessels by inhibition of VEGF signaling did not prevent anti-E-cadherin antibodies from reaching their target on tumor cells even in regions lacking CD31-positive blood vessels. How did the antibody move from sites of extravasation to tumor cell clusters? After VEGF inhibition, factors that may influence antibody distribution include (a) reduced interstitial fluid pressure, which increases convective driving force for antibody extravasation (21); (b) increased antibody accumulation per normalized tumor vessel, which partially offsets the effect of tumor vessel regression; and (c) increased number of empty sleeves of basement membrane, which facilitate the transport of extravasated antibodies in tumor stroma. Together, these factors favor the continued delivery of therapeutic antibodies that recognize epoepitopes on tumor cells after the tumor vasculature is pruned by angiogenesis inhibitors.

Conclusions

Fluorescence and confocal microscopic imaging of RIP-Tag2 tumors revealed that extravasated antibodies accumulate in irregular patchy networks of tumor stroma. Antibody distribution matches the distribution of fibrin and fits better with the pattern of basement membrane than with blood vessels where extravasation occurs. Unlike other antibodies tested, anti-E-cadherin antibody, which targets tumor cell epitopes, accumulates within tumor cell clusters as well as in stroma. Inhibition of VEGF signaling decreases the overall distribution of extravasated antibodies, in large part due to reduced tumor vascularity. However, distribution expressed per surviving vessel actually increases, probably due to increases in driving force for leakage and transport after normalization of tumor vessels and interstitium. Antibodies accumulate in sleeves of basement membrane left behind by regressing tumor vessels. These may serve as preferential distribution routes in tumors. Maintenance of delivery of anti-E-cadherin antibody to tumor cells indicates the potential for complementary actions of angiogenesis inhibitors and therapeutics targeted to tumor cells.

Acknowledgments

Received 3/21/2005; revised 10/21/2005; accepted 11/15/2005.

Grant support: NIH grants HL-54136 and HL-79157 from the National Heart, Lung, and Blood Institute (D.M. McDonald), NIH grants CA-82923 and P50-CA-90270 from the National Cancer Institute, a grant from Pfizer Global Research and Development, San Diego, and funding from AngelWorks Foundation and the Vascular Mapping Project.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Tomomi Kamba, Peter Baluk, Michael Mancuso, Tom Le, Barbara Sennino, and Rachel Davis for expert technical help and discussions; Ian Kasman for his help with confocal microscopic imaging; Douglas Hanahan for supplying breeding pairs for our colony of RIP-Tag2 mice; Wei Wei for genotyping the mice; and Pfizer Global Research and Development, San Diego, for supplying AG-013736.

References


Effect of Inhibition of Vascular Endothelial Growth Factor Signaling on Distribution of Extravasated Antibodies in Tumors

Tsutomu Nakahara, Scott M. Norberg, David R. Shalinsky, et al.