The Addition of AG-013736 to Fractionated Radiation Improves Tumor Response without Functionally Normalizing the Tumor Vasculature

Bruce M. Fenton and Scott F. Paoni
Department of Radiation Oncology, University of Rochester Medical Center, Rochester, New York

Abstract
Although antiangiogenic strategies have proven highly promising in preclinical studies and some recent clinical trials, generally only combinations with cytotoxic therapies have shown clinical effectiveness. An ongoing question has been whether conventional therapies are enhanced or compromised by antiangiogenic agents. The present studies were designed to determine the pathophysiologic consequences of both single and combined treatments using fractionated radiotherapy plus AG-013736, a receptor tyrosine kinase inhibitor that preferentially inhibits vascular endothelial growth factor receptors. DU145 human prostate xenograft tumors were treated with (a) vehicle alone, (b) AG-013736, (c) 5 × 2 Gy/wk radiotherapy fractions, or (d) the combination. Automated image processing of immunohistochemical images was used to determine total and perfused blood vessel spacing, overall hypoxia, pericyte/collagen coverage, proliferation, and apoptosis. Combination therapy produced an increased tumor response compared with either monotherapy alone. Vascular density progressively declined in concert with slightly increased α-smooth muscle actin–positive pericyte coverage and increased overall tumor hypoxia (compared with controls). Although functional vessel endothelial apoptosis was selectively increased, reductions in total and perfused vessels were generally proportionate, suggesting that functional vasculature was not specifically targeted by combination therapy. These results argue against either an AG-013736- or combination treatment–induced functional normalization of the tumor vasculature. Vascular ablation was mirrored by the increased appearance of dissociated pericytes and empty type IV collagen sleeves. Despite the progressive decrease in tumor oxygenation over 3 weeks of treatment, combination therapy remained effective and tumor progression was minimal. [Cancer Res 2007;67(20):9921–8]

Introduction
Although antiangiogenic strategies have been shown highly promising in preclinical studies and some recent phase II and III clinical trials (1), generally only combinations of antiangiogenic and cytotoxic therapies have shown clinical effectiveness. As recently reviewed (2), an ongoing question about such combined therapies has been whether conventional therapies, such as radiotherapy or chemotherapy, are enhanced or compromised by antiangiogenic agents. Although antiangiogenic strategies might be expected to destroy tumor vasculature and thereby deprive the tumor of radiosensitizing oxygen or hinder access to chemotherapeutic agents, results are inconclusive. Some workers have found decreased tumor oxygenation and blood flow following such agents (3, 4), whereas others have shown the opposite (5, 6). Concurrent (4), postradiotherapy (7, 8), and preradiotherapy (9, 10) combination scheduling with antiangiogenics have all been shown advantageous (11).

An intriguing hypothesis recently reviewed by Jain (12) contends that vascular endothelial growth factor (VEGF) inhibitors can selectively prune the chaotic and inefficient vasculature commonly found in tumors, resulting in a transiently normalized vascular configuration. This improved vasculature, characterized by reduced vessel counts and increased coverage of periendothelial support cells, or pericytes, is thus predicted to more efficiently deliver both oxygen and drugs. A second often quoted hypothesis is that the susceptibility of established tumor blood vessels to interference with VEGF/VEGF receptor-2 (VEGFR-2) signaling may be limited to vessels that lack pericyte coverage (13–15). Recent studies, however, have noted that pericytes can also be actively recruited before pruning (6).

The present investigation was designed to evaluate the consequences of combining fractionated radiation with AG-013736, a potent receptor tyrosine kinase inhibitor of VEGFRs that also inhibits platelet-derived growth factor receptors (PDGFR) at higher doses. Does vascular density decrease following 1 to 3 weeks of single or combination treatment, and if so, does tumor hypoxia increase or instead decrease in line with vascular normalization? Given the well-known dependence of radiosensitivity on tumor oxygen levels, any increase in hypoxia would presumably also compromise further combination therapy. Our second objective was to compare alterations in the tightness and coverage of two pericyte markers and to determine the relation between pericyte coverage and selective vascular ablation. PDGFRβ was selected as a marker of perivascular progenitors or less mature pericytes (16), whereas α-smooth muscle actin (α-sma) was chosen to distinguish more mature pericytes or vascular smooth muscle cells (16, 17).

The current findings argue against a functional normalization of the tumor vasculature following combination therapy. Although treatment reduced vascular densities, overall tumor hypoxia progressively increased. Despite this decrease in oxygenation, however, combination therapy remained effective and tumor growth was inhibited.

Materials and Methods
Tumor and animal models. The DU145 human prostate carcinoma cell line was obtained from the American Type Culture Collection and maintained in DMEM (Mediatech-Celgro) supplemented with 10% fetal bovine serum. Viable tumor cells (10⁶) were implanted into the left hind legs.
of NCr nu/nu male mice and grown to tumor volumes of 200 to 400 mm³. Tumor (including leg) diameters were measured thrice weekly using a graduated hole template, with opposite nontumor leg diameters subtracted to calculate actual tumor volumes: tumor volume = π / 6 × (tumor leg diameter³ − nontumor leg diameter³). Mice were housed in microisolator cages and given food and ad libitum. Guidelines for the humane treatment of animals were followed as approved by the University Committee on Animal Resources.

Treatments. AG-013736, a receptor kinase inhibitor of VEGFRs and, at higher doses, PDGFRs (IC₅₀ = 0.1 nmol/L for VEGFR-1, 0.2 nmol/L for VEGFR-2, 0.1–0.3 nmol/L for VEGFR-3, and 1.6 nmol/L for PDGFR; ref. 18), was provided by Pfizer Global Research and given once daily by gavage in a 152Cs source operating at 2.4 Gy/min. Mice were confined to plastic jigs with tumor-bearing legs extended through an opening in the side, allowing local irradiations. Fractionated doses were given in five daily 2 Gy fractions per week (omitting weekends). For combination treatments, radiotherapy was delivered first, and AG-013736 was given within 4 h. Mice were sacrificed, and tumors were excised and then quickly frozen (using liquid nitrogen) following 1, 2, or 3 weeks of treatment.

DiOC₆, perfusion marker and EF₅ hypoxia marker. To visualize blood vessels open to flow, DiOC₆, an intravascular stain that preferentially stains cells immediately adjacent to the vessels, was injected intravascularly by tail vein 1 min before freezing (19). Localized areas of tumor hypoxia were assessed in 9-µm frozen sections (one section taken as near as possible to the tumor center for each combination of stains) by immunohistochemical identification of sites of 2-nitroimidazole metabolism (20). A pentfluorinated derivative of etanidazole (EF₅) was injected intravascularly (0.2 mL of 10 mmol/L EF₅) 1 h before tumor freezing (21) followed by a second dose 45 min later. Regions of high EF₅ metabolism were visualized using a Cy3 fluorochrome (Amershams) conjugated to the ELK3-51 antibody, which is extremely specific for the EF₅ adducts that form when the drug is incorporated by hypoxic cells (22). Both the EF₅ (made by the National Cancer Institute) and the ELK3-51 were obtained from the University of Pennsylvania Imaging Service Center (C. Koch, Director).

Immunohistochemistry and image acquisition. Tumor sections were imaged using a 10× objective (Olympus BX40 microscope), digitized (QImaging Retiga 1300C Peltiler-cooled, 12-bit digital camera), background corrected, and image analyzed using Image-Pro software (Media Cybernetics). Twelve-bit gray-scale image montages from 16 adjacent microscope fields (encompassing a total area of 21.6 mm²) were automatically acquired and digitally combined for multiple stains (20). First, images of the DiOC₆ were obtained immediately after cryosectioning and fixing in cold acetone for 10 min. Following staining, this section was returned to identical stage coordinates to obtain images of both EF₅/Cy3 and an endothelial cell marker [MECA-32 (biotinylated primary, 1:100; PharMingen) followed by incubation with Vectastain ABC Elite Standard kit and AEC detection (Vector Laboratories)].

Adjacent frozen sections were sliced to visualize combinations of antibodies, generally dual staining of endothelial cells (MECA-32) versus markers for either (a) proliferation [Ki-67 (MIB-1 clone, mouse anti-human, 1:150; Dako) in conjunction with the M.O.M. "mouse primary antibody on mouse tissue" kit (Vector Laboratories)], (b) apoptosis [FITC-terminal deoxynucleotidyl transferase–mediated diUTP nick end labeling (TUNEL) assay, DeadEnd Fluorometric TUNEL System (Promega), with the following modifications: sections were fixed in 4% neutral buffered formalin for 10 min after panendothelial cell antigen staining, washed in PBS, and then incubated for 15 min with 0.2% Triton X-100 before apoptosis staining], (c) pericytes [PDGFRβ, clone AP5B, rat anti-mouse, 1:50 (eBioscience) or α-sm, clone LA4, mouse Cy3 directly conjugated, 1:2000 (Sigma), or (d) vascular basement membrane (type IV collagen, polyclonal rabbit anti-mouse, 1:2000 Chemicon). For Ki-67 and apoptosis, 9-µm sections were fixed in cold acetone for 10 min, air dried for 10 min, and stored at ~80°C. For pericytes, 20-µm sections were fixed for 10 s in 1% neutral buffered formalin, washed and covered in PBS, imaged for DiOC₆, PBS aspirated, fixed in room temperature acetone for 1 min, and air dried for 30 min before staining. For type IV collagen, 20-µm sections were fixed in warm acetone for 1 min and air dried for 30 min. Secondary antibody detection was done using species-specific Alexa Fluor 488 (type IV collagen) or 546 (Ki-67, PDGFRβ); 1:500; Molecular Probes) combined with MECA-32 using contrasting Alexa Fluor secondary antibodies. Sections were coveredslipped with Slow Fade Gold antifading agent (Molecular Probes).

Image analysis: vascular spacing, apoptosis, and proliferation. As described previously (25), tumor blood vessel spacing was determined using a combination of image segmentation and distance map filtering to obtain a spatial sampling of distance filter intensities, which are directly proportional to the distribution of distances to the nearest vessel. These distances (dependent on tumor blood vessel spacing) are reflective of the median distances over which oxygen and nutrients must diffuse to reach all cells of the tumor.

Colocalized and thresholded images of MECA-32 endothelial cell staining, DIOC₆, TUNEL, and Ki-67 staining were obtained, and percentage area was determined for each as well as for the overlap between TUNEL and perfused vessel staining (using custom Image-Pro macros). Percentage apoptotic vessel overlap was calculated by dividing the area of vessel apoptosis overlap by the perfused or total vessel area.

Statistical analysis. Treatments were compared using Student’s t test or the Mann-Whitney rank sum test and considered significant for P ≤ 0.05.

Results

Effects of AG-013736 and/or fractionated radiotherapy on tumor growth. Mice bearing DU145 tumors were first treated with 10, 25, or 50 mg/kg/d AG-013736 to define a dose that would slow but not stop tumor growth, thus allowing the later effects of combination therapies to be recognized. Tumor volumes were measured thrice weekly, mice were sorted into groups having roughly equal mean volumes, and treatments were initiated at volumes between 200 and 400 mm³. Figure 1A presents percentage tumor volume increase as a function of AG-013736 dose. Tumor responses following doses of 10 and 25 mg/kg (by gavage) were roughly equivalent, but 50 mg/kg resulted in substantial inhibition of tumor growth over the 3 weeks of treatment. A dose of 25 mg/kg was therefore selected for the combination experiments. Based on pharmacokinetic data, once daily dosing of AG-013736 at 25 mg/kg only provided transient (2–4 h per day) inhibition of PDGFRβ (data not shown). Preliminary studies also showed that a schedule of 5 × 2 Gy/wk of radiation slowed tumor growth to a rate similar to that following 25 mg/kg AG-013736 (Fig. 1B).

Mice were next treated for a period of 2 weeks with either fractionated radiation (5 × 2 Gy/wk) or AG-013736 (25 mg/kg/d) and 1 to 3 weeks for the combination. As shown in Fig. 1B, tumor volume at the end of 2 weeks was significantly reduced for either single or combination treatments. Percentage increases in tumor volume were similar between radiotherapy (40 ± 9.8%) and AG-013736 (48 ± 9.2%), and the combination was markedly reduced versus controls (12 ± 5.7% versus 77 ± 11%; P < 0.001). A separate experiment measured combination and control volumes over 3 weeks of treatment, at which time the percentage increases in tumor volume were 23.3 ± 14% and 211 ± 57%, respectively (P < 0.001).
Total and perfused blood vessel spacing increases following either single or combination treatments. We next examined the effects of treatment on total and perfused vascular spacing to gauge whether radiotherapy, AG-013736, or the combination affected tumor vascular spacing. As shown in Fig. 2A, 2 weeks of AG-013736, radiotherapy, or the combination significantly increased both total (black columns) and perfused (white columns) blood vessel spacing, although none of the three treatments was significantly different from each other. In this and the following figures, treated tumors could have been compared with either day-matched or volume-matched controls. To minimize pathophysiologic variations due to tumor growth alone, our usual approach is to favor the volume-matched comparisons. Thus, week 1 controls (mean volumes = 560 ± 30 mm³) were chosen for comparison.
because they were a fairly close match to all of the week 2 treated tumor volumes (which ranged from $350 \pm 30$ mm$^3$ for the combination-treated tumors to $530 \pm 60$ for the AG-013736). It should be noted, however, that in the case of this relatively slow-growing DU145 tumor model, vessel spacing and hypoxia did not change significantly for the control tumors over the range from 560 to 850 mm$^3$. Figure 2B compares week 1 controls to combination-treated tumors frozen at 1, 2, or 3 weeks. Although the combination produced significant increases in total and perfused vessel spacing at most time points in relation to week 1 controls, changes were minimal at week 1, most pronounced at week 2, and less pronounced by week 3 (which were not significantly different from week 2 for either total or perfused).

**Tumor hypoxia increases following combination therapy.** Overall tumor hypoxia was quantified in frozen sections based on the intensity of a fluorescently conjugated antibody to EF5. Perfused spacing increased substantially at week 2 for all three treatments (Fig. 2A), but hypoxia was unchanged for the radiotherapy ($P = 0.29$) and increased only minimally for either AG-013736 ($P = 0.011$) or the combination ($P = 0.033$), which were no different from each other ($P = 0.35$). Overall hypoxia increased slightly at weeks 1 and 2 in combination-treated tumors ($P = 0.004$ and 0.002, respectively; Fig. 2D) and substantially at week 3 ($P < 0.001$). Thus, although tumor volumes remained essentially constant following combination therapy, vascular functionality steadily decreased with an ultimate increase in tumor hypoxia.

Although alterations in hypoxia might be expected to parallel changes in perfused vessel spacing, this was not always the case. For instance, perfused vessel spacing increased substantially between weeks 1 and 2 of treatment, whereas overall tumor hypoxia remained constant. At week 3, perfused spacing was not significantly different from weeks 1 or 2 ($P = 0.063$), but hypoxia markedly increased ($P = 0.003$). As has been shown previously using direct measures of tumor microvessel HbO$_2$ saturations (24), vessels defined as "perfused" can vary markedly in terms of their functionality. Thus, the constant levels of perfused vessel spacing over the 3 weeks of treatment may correspond to a progressive decrease in functionality with continued treatment, leading to the observed increase in hypoxia. We therefore consider the EF5 intensities as the more rigorous assay for changes in overall tumor hypoxia following treatment. This index is incapable of distinguishing between clonogenic and nonclonogenic tumor cell subpopulations, however, which can only be definitively defined using survival or clonogenic assays of "radiobiological hypoxic fraction" (25).

**Combination therapy increases perfused vessel endothelial apoptosis and reduces tumor cell proliferation.** Based on TUNEL staining, neither overall tumor cell apoptotic density nor endothelial cell apoptosis was significantly increased by either single or combination treatments (data not shown). Apoptosis of perfused vessel endothelial cells (Fig. 3A and B) increased significantly for combination-treated tumors at weeks 2 and 3 ($P = 0.03$ versus 0.097 for radiotherapy alone and 0.201 for...
AG-013736 alone). This could reflect increased drug and oxygen concentrations within the vessels, assuming that these vessels were also perfused at time of treatment. No significant differences were noted between combination treatments and either radiotherapy or AG-013736 alone (P = 0.22 or 0.66, respectively). Finally, based on percentage positive Ki-67 staining, tumor cell proliferation was significantly increased by radiotherapy at 2 weeks after therapy (Fig. 3C) but decreased by the combination at both 2 and 3 weeks (Fig. 3D). Again, the combination was not significantly different from AG-013736 alone.

**PDGFR**β pericyte dissociation, but not coverage, is increased by combination treatment. PDGFRβ+ is generally believed to mark a progenitor population of pericytes, whereas α-smα− denotes more mature cells (16). Figure 4 presents effects of treatment on (a) percentage PDGFRβ+ coverage (i.e., the fraction of blood vessel area covered by PDGFRβ+ pericytes) and (b) percentage dissociation (the fraction of PDGFRβ+ pericyte area not overlapping blood vessels). In the current study, vascular coverage of PDGFRβ+ pericytes was ~75% in controls and was not significantly altered by single or combined treatments (Fig. 4A and B, yellow columns). Dissociation of PDGFRβ+ pericytes, however, increased progressively for radiotherapy, AG-013736, and the combination (Fig. 4C) and remained elevated throughout the 3 weeks of combination therapy (Fig. 4D). Dissociation in the combination tumors was significantly higher than for the AG-013736 (P = 0.014), which in turn was significantly higher than for the radiotherapy (P = 0.038). Interestingly, dissociation in response to the combination peaked at week 2, which corresponds to the peak in vascular spacing (Fig. 2B) and suggests a possible link between vascular ablation and pericyte dissociation. Representative pseudocolor images of tumors dual stained for endothelial cells and both PDGFRβ and α-smα− pericytes are shown in Fig. 4E.

α-smα− pericyte dissociation and coverage are increased by combination treatment. Coverage of α-smα− pericytes was substantially lower than for PDGFRβ+ pericytes in untreated tumor vessels (~20% versus ~75%) but increased following both radiotherapy or the combination and remained elevated throughout 3 weeks of therapy (Fig. 4A and B, blue columns). This suggests either (a) selective ablation of non-α-smα− pericyte-coated blood vessels or (b) recruitment of α-smα− pericytes to previously non-coated vessels. Because vessel numbers were also substantially decreased at these times, selective ablation seems the more likely mechanism after week 1 of combination therapy. At weeks 2 to 3, however, vessel counts were further reduced in the absence of increased α-smα− coverage (Fig. 4B, blue columns), suggesting that vessels are then similarly targeted irrespective of pericyte coverage. As with PDGFRβ+ cells, dissociation of α-smα− cells increased significantly with both mono and combined therapies and progressively increased with increasing weeks of combination therapy (Fig. 4C and D, blue columns).

Type IV collagen–coated basement membranes are retained following endothelial ablation. Figure 5 illustrates changes in percentage areas of endothelial cells, PDGFRβ+ pericytes, α-smα− pericytes, and type IV collagen (used to identify vascular basement membranes) following combination treatment. As expected based on the increased vascular spacing results of Fig. 2B, percentage area of endothelial cells decreased significantly in relation to controls at each time point (P < 0.001 in each case). In contrast, no significant changes were seen for type IV collagen, α-smα−, or PDGFRβ percentage areas. This suggests that the net effect of the combination therapy was to ablate the endothelial cell layer of the blood vessels while retaining the basement membrane sleeve and perivascular cells, as has been previously reported following AG-013736 alone (26). In control tumors, dual staining revealed extensive overlap between type IV collagen− basement membrane and endothelial cell staining (Fig. 5B, yellow), although small subregions with high proportions of disassociated collagen sleeves were also sometimes present. For combination-treated tumors, in contrast, vascular counts were substantially reduced and empty collagen sleeves were broadly distributed throughout the tumor cross-section (Fig. 5C). Finally, overlap between PDGFRβ+ and collagen was also substantially reduced following combination therapy (compare Fig. 5D and E), suggesting that treatment results in the dissociation of basement membrane sleeves from pericytes as well as endothelial cells.

**Discussion**

The current studies are the first to treat tumors with a combination of fractionated radiotherapy plus AG-013736, a receptor tyrosine kinase inhibitor of primarily VEGFRs at the dose levels used in this study. This combination resulted in a significantly reduced tumor growth rate in comparison with either monotherapy alone. For the combination-treated tumors, tumor vascular density significantly decreased and hypoxia progressively increased compared with controls but was not significantly different from either radiotherapy or AG-013736 at the week 2 time point. Functional vessel endothelial cell apoptosis was also increased by the combination. However, differences with respect to either monotherapy were again minimal. Because alterations in total and perfused vessel spacings were generally proportionate, functional vasculature was likely not selectively targeted by the combination therapy.

Previous work has suggested that VEGFR-2 inhibitors alone can lead to vascular normalization, which has been defined as a combination of selective pruning of excess tumor vessels, an increase in pericyte coverage, and a transient improvement in tumor oxygenation (6, 27). In agreement with the current results, however, alternate studies have shown short-term and long-term impairment of vascular function and increased tumor hypoxia following VEGFR-2 inhibition (3). As summarized in recent reviews (11, 28), combinations of radiotherapy with antiangiogenic strategies have almost universally shown increased tumor growth delay when compared with radiotherapy alone. Because neither irradiated nor unirradiated tumor cells are usually affected by antiangiogenic compounds in vitro (11), this suggests a radiosensitizing effect that is either restricted to the endothelial cells, related to alterations in tumor blood flow, or both. Endothelial sensitization is supported by the work of Schueneman et al. (29), who administered daily radiotherapy after SU11248, a similar multitargeted small-molecule inhibitor of VEGFRs and PDGFRs, and found that the combination reduced tumor blood flow but increased endothelial-specific apoptosis and vascular destruction. SU11657, another inhibitor of VEGFRs and PDGFRs, was most effective when single-dose radiotherapy was delivered 1 day after the drug. At that time, tumor interstitial pressures were substantially reduced and tumor blood flow was presumably increased (9).

As a rule, pericytes are believed to form tighter associations with endothelial cells in normal tissue compared with tumors (17, 30). However, the literature has been somewhat conflicted in defining optimal antibodies for identification of pericyte maturity. A major obstacle is the variability in marker expression and coverage among different tumor models (13, 17, 31), although elegant studies
have convincingly shown that PDGFRβ+ cells are a progenitor of α-sma+ perivascular cells (16) and that vessels covered by more mature α-sma+ pericytes are selectively protected in tumors subjected to VEGF withdrawal (13). Previous work has also suggested that pericyte coverage does not generally increase following radiotherapy alone (32) and can either increase (6) or decrease (16) following antiangiogenic strategies. Although pericytes can confer resistance to a variety of VEGFR-targeting agents, the inclusion of specific PDGFR inhibitors has been shown to overcome this resistance, leading to vessel destabilization and regression (15). Recent work suggests that the effects of AG-013736 are most likely predominantly due to VEGFR rather than PDGFRβ inhibition (26). In spontaneous pancreatic tumors, AG-013736 was shown to produce a tightening of α-sma+ pericyte coverage combined with a loss of both pericyte-coated and pericyte-free vessels (30). Vascular area was found to decrease by 79%, but α-sma+ pericyte area was only reduced 33%, suggesting a preferential targeting of non-α-sma+ vessels. PDGFRβ+ pericytes were unchanged following administration or withdrawal of AG-013736, and vessels that survived treatment were generally perfused.

Figure 4. Effect of treatment on pericyte % coverage and dissociation. A, effect of 2 wk of treatment (2 Gy radiotherapy plus 25 mg/kg AG-013736 per day) on % coverage (defined as the ratio of area overlap between endothelial cells and pericytes divided by the total area of endothelial cells) for PDGFRβ+ (yellow columns) and α-sma+ (blue columns). B, effect of 1 to 3 wk of combination treatment on % coverage. C, effect of 2 wk of treatment on % dissociated pericytes (defined as the ratio of the area of pericytes not overlapping with endothelial cells divided by the total pericyte area). Columns, mean; bars, SE. *, significant differences of treated tumors in relation to week 1 controls (P < 0.05). D, effect of 1 to 3 wk of combination treatment on % dissociated pericytes. E, representative thresholded, pseudocolor images of α-sma+ (red) or PDGFRβ+ pericytes (red), MECA-32 endothelial marker (green), and overlap (yellow). Bar, 100 μm.
This contrasts somewhat with current findings in which perfused and nonperfused vessels were nonselectively ablated by combination treatments. However, we did observe an initial increase in \( \alpha \)-sma\(^+\) coverage, combined with increased dissociation of both \( \alpha \)-sma\(^+\) and PDGFR\(\beta\)\(^+\) pericytes. This supports a selective ablation of non-\( \alpha \)-sma\(^+\)-coated vessels rather than a recruitment of \( \alpha \)-sma\(^+\) pericytes because vascular counts also substantially decreased. This selectivity was lost by week 2 of treatment, however, and further vascular reductions were not mirrored by corresponding alterations in pericyte coverage. Instead, a significant dissociation of both \( \alpha \)-sma\(^+\) and PDGFR\(\beta\)\(^+\) pericytes was observed following either AG-013736 or the combination.

Recent studies have also suggested that basement membrane sleeves are retained following endothelial destruction and can later provide a scaffolding for rapid vascular regrowth following cessation of VEGF inhibition (26). We found a similar dissociation of type IV collagen vascular sleeves from endothelial cells as well as from PDGFR\(\beta\)\(^+\) pericytes following combination therapy. Interestingly, the timing of increases in PDGFR\(\beta\) dissociation precisely paralleled the temporal reductions in vessel counts following both single and combined therapies. Together, these data suggest that the net effect of the combination treatment is to selectively destroy endothelial cells while at the same time stripping pericytes from their associated basement membranes.

In the current experimental design, combination therapy was scheduled such that AG-013736 was delivered after radiotherapy under the assumption that pretreatment with the antiangiogenic agent would reduce tumor perfusion and thereby compromise radiotherapy (8). AG-013736 could alternatively have been administered just before radiotherapy, however, which would presumably further accentuate endothelial apoptosis and sensitization of endothelial cells to radiotherapy. Although previous studies have reported increased endothelial and tumor cell apoptosis immediately following radiotherapy, both return to baseline levels by 1 and 2 weeks (32). Our weekly time points could conceivably have missed transient changes, which illustrates the importance of including multiple time points.

In conclusion, the current findings argue against a treatment-induced functional normalization of the tumor vasculature when applying combination therapy. Rather than tightening pericytes, AG-013736 and the combination treatment served instead to loosen pericyte-vessel and pericyte-basement membrane associations in this tumor model. Treatment substantially reduced total and functional vascular densities, but overall tumor hypoxia progressively increased, in contrast to the normalization hypothesis. Despite the reduction in oxygenation, tumor progression was minimal over 3 weeks of combination treatment, most likely due to continued vascular destruction and the prevention of new vessel growth. Further studies are essential to extend these measurements to additional tumor models and to determine whether alternative scheduling may also enhance treatment response.

Acknowledgments

Received 3/21/2007; revised 7/16/2007; accepted 7/27/2007.

Grant support: Department of Defense grant W81XWH-04-1-0827 and National Cancer Institute grant CA52586.

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 Drs. David Shalinsky and Dana Hu-Lowe (Pfizer Global Research, La Jolla, CA) for providing the AG-013736 small-molecule inhibitor and P. Sabrina Agro for technical assistance.

References


The Addition of AG-013736 to Fractionated Radiation Improves Tumor Response without Functionally Normalizing the Tumor Vasculature

Bruce M. Fenton and Scott F. Paoni


Updated version  Access the most recent version of this article at: [http://cancerres.aacrjournals.org/content/67/20/9921](http://cancerres.aacrjournals.org/content/67/20/9921)

Cited articles  This article cites 32 articles, 13 of which you can access for free at: [http://cancerres.aacrjournals.org/content/67/20/9921.full.html#ref-list-1](http://cancerres.aacrjournals.org/content/67/20/9921.full.html#ref-list-1)

Citing articles  This article has been cited by 4 HighWire-hosted articles. Access the articles at: [http://cancerres.aacrjournals.org/content/67/20/9921.full.html#related-urls](http://cancerres.aacrjournals.org/content/67/20/9921.full.html#related-urls)

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.