INTRODUCTION

Local recurrence of malignancies after treatment in up to 55% of cases (1–3) presents a significant problem in cancer management. It is known that most tumors require plentiful blood supply to provide nutrients to metabolically demanding tumor cells and that tumor angiogenesis depends on complex interactions of vascular growth factors and their endothelial cell-bound receptors (4–8). However, the well-studied mechanisms that are mobilized for neoangiogenesis of tumors may not necessarily be those activated in post-treatment recovery. In the context of tumor regrowth, up-regulation of angiogenic growth factor expression (by tumor cells or endothelium) and of endothelial growth factor receptors is likely. Resistance to subsequent treatment (9) may be attributable, in part, to constitutive overproduction of angiogenesis-stimulating growth factors and receptors by tumor cells and tumor vessels, and may serve as a suitable target for therapy after standard cytotoxic treatment.

Tumor cells have been shown to stimulate angiogenesis, migrate toward host vessels, and proliferate around host vasculature, and so they, perhaps more than stromal cells, are most likely active in stimulating angiogenesis after cytotoxic treatment (10, 11). It is known that VEGF3 receptor-ligand interactions, common targets of antiangiogenic therapy (12), induce differentiation and proliferation of endothelial cells (13, 14) during vasculogenesis, establishment of tumors, and in wound healing. However, other secreted angiogenic growth factors, including PIGF and the angiopoietins, ang-1 and ang-2, might also play a significant role during tumor recovery. PIGF, an alternate ligand to VEGF receptor flt-1, induces endothelial cell proliferation and differentiation (2, 3, 15–22). ang-1 influences interactions between the endothelium and surrounding matrix (20–22). ang-2 disrupts endothelial cell/pericyte contacts and promotes angiogenesis when VEGF is present (7). The angiogenic pathways controlled by the receptor for ang-1 and -2 (Tie-2) are distinct from each other and from VEGF receptor pathways (23, 24). The orphan receptor Tie-1 is essential for maintaining endothelial cell integrity and formation of mi-
cavernous endothelium, and, therefore, may be significant in post-treatment recovery of solid tumors (24).

To determine the angiogenic growth factor pathways induced in tumors after antitumor therapy and before relapse, RAIT, a well-documented and often successful experimental cytotoxic cancer therapy (25), was used to treat mice with well-established human xenograft tumors. RAIT was administered by injection of a single dose of 131I-labeled anti-CEA or anti-EGP-1 antibodies (26, 27). CEA is an antigen commonly expressed by human carcinomas (including the colon tumors used in this study) and has been used for diagnostic imaging of colon tumors (26). Radiation was delivered to human lung adenocarcinoma xenograft Calu-3 by antibody RS-73G11, an antibody against EGP-1, that is internalized on binding to EGP-1 (27).

The cytotoxic effect of RAIT arrests tumor growth for 3–5 weeks, but often tumors regrow after 6 weeks. Four tumors of varied responsiveness to RAIT were examined up to 6 weeks after treatment for production of VEGF, PI GF, flk-1 and flt-1, ang-1 and -2, and Tie1 and -2 by IHC, immunoblot (VEGF only), or comparative RT-PCR (PIGF only). IHC was chosen as the main tool of analysis, because it enables local or specific expression to be pinpointed and documented, e.g., tumor cell versus stromal cell versus perivascular regions. In addition, changed intensity of staining denotes changes in expression so that up- or down-regulation can be documented semiquantitatively.

Hypoxia, present in tumors because of metabolic activity or in treated tumors as a result of treatment-induced damage, has been shown to increase VEGF, flt-1, and flk-1 expression (28–31). Therefore, tumor hypoxia was also assessed by IHC for pimonidazole adducts before and after treatment to determine possible correlations with induction or up-regulation of endothelial receptor or ligand expression.

MATERIALS AND METHODS

Tumors and Treatment of Mice. Human tumor lines HT-29, LoVo, and Calu-3 were purchased from American Type Culture Collection (Bethesda, MD). HT-29 and LoVo are colon adenocarcinomas. GW-39, first established in 1966 from a human signet ring cell colon carcinoma (32), is perpetuated by serial transplantation in animals. Calu-3 is a lung adenocarcinoma isolated from pleural effusion.

HT-29, LoVo, and Calu-3 were cultured in vitro according to recommendations from American Type Culture Collection, and 10⁷ cells were implanted s.c. in the flanks of nude mice; 300 µl of a 10% cell suspension of GW-39 tumor tissue was implanted s.c. in the flanks of nude mice. When tumors were ~0.5 cm³ in volume (~2 weeks after implantation), RAIT was commenced with one i.v. injection of radiolabeled antibody representing the MTD for that antibody. The colonic tumors HT-29, GW-39, and LoVo were treated with 240 µCi of 131I-labeled mAb MN-14 anti-CEA (Immunomedics, Inc., Morris Plains, NJ; Ref. 26). Calu-3 was treated with 295 µCi 131I-RS-7-3G11, an anti-EGP-1 that is internalized when bound to its ligand (27). Although the MTD was used, each tumor received different doses of radiation (cGy) based on biodistribution data (33). Calculated dose for each tumor: LoVo 2195 cGy; HT-29 1140 cGy; GW-39 3830 cGy; and Calu-3 1500 cGy. UT (n = 3–11/ time point/experiment) and RAIT-treated tumor samples (n = 3–5/time point/experiment) were harvested at 1-week intervals until 6 weeks after RAIT, when tumors typically show signs of regrowth. Results presented are from two independent experiments except for HT-29 VEGF IHC (three experiments) and Calu-3 (one experiment). Results of repeat experiments were similar.

IHC. To measure regional expression of various markers, paraffin-embedded samples were cut into 5 µm (u) sections; deparaffinized, and then rehydrated. Sections were blocked with appropriate normal serum in PBS, incubated with either anti-VEGF (mAb or rabbit polyclonal), -flk-1, -flt-1, -tie-1, and -2 (all rabbit polyclonal), ang-1 and -2 (goat polyclonals; all from Santa Cruz Biotechnology Inc., Santa Cruz, CA), or irrelevant negative antibody controls. After incubation for 45 min, appropriate secondary antibody was applied for 30 min followed by immersion in 3% H₂O₂ (5 min) to quench endogenous peroxidases. Avidin-biotin-HRP conjugate was applied to washed slides for 30 min. Slides were incubated 10 min with HRP substrate, 3,3′-diaminobenzidine (Sigma Chemical Co., St. Louis, MO or Santa Cruz Biotechnology) or True Blue (KPL, Gaithersburg, MD), and counterstained with hematoxylin for 3,3′-diaminobenzidine (Sigma Chemical Co.) or Nuclear Fast Red (True Blue; Vector, Burlingame, CA).

Stained slides were examined at 100× and 400×, and rated for intensity of staining by the following standard: faint (0.25–0.75), moderate (>0.75–1.5), heavy (>1.5–2.5), and intense (>2.5–3); scoring was in 0.25-point increments. All of the slides were read (blinded) by one researcher and spot-checked by another. Only viable-appearing areas of the tumors were assessed (>5 fields/sample), and a value representing the overall staining intensity and stained area was assigned to each slide. Extracellular, necrotic areas, and WBCs were not included in the scores. Background staining (indicated by control antibody) was also assessed and then subtracted. Scores shown in the figures are the mean of samples from each experimental group for each factor or receptor. Staining was broken down into tumor cell and perivascular areas of the tumors. The range of values for UT controls for each time point (n = 3–11) is represented as a horizontal bar in Fig. 1, A, Fig. 2, and Fig. 4. When UT controls were available for IHC beyond week 1, they usually exhibited <2-fold change in expression of growth factors or their receptors over time except for LoVo tumor cell VEGF.

Changes in tumor cell production of growth factors VEGF, PI GF, ang-1, and ang-2 are represented in Figs. 1 and 4. Perivascular staining for growth factors, if different from UTs, is reported in the text of the “Results” section. Only perivascular expression of growth factor receptors is represented in Fig. 2. The large size of UT control tumors usually necessitated sacrifice of tumor-bearing animals before week 6.

IHC for Hypoxia. Mice were treated 3 h before tumor harvest with 100 mg/kg of hypoxic, viable cell targeting pimonidazole HCl i.p. (Hypoxyprobe-1; NPI, Inc., Belmont, MA; Ref. 34). Tumors were harvested, preserved in formalin, and paraffin-embedded. Presence of pimonidazole adducts was detected in tumor tissue by IHC using Hydroxyprobe1Mab1, a
mouse monoclonal IgG1 (NPI, Inc.). The recommended procedure for IHC from the supplier of mAb was followed.

Hypoxic staining was assessed as above, including stain intensity, percentage of the tumor section stained above background, and which areas of the tumor were most intensely stained (e.g., those adjacent to necrosis).

**Immunoblot.** To detect total tumor VEGF, tumor samples were extracted in the following buffer: 10 mM Tris (pH 7.6), 1% Triton X-100, 150 mM NaCl, 5 mM EDTA, and the following protease inhibitors: phenylmethylsulfonyl fluoride (1 μM), leupeptin (10 μg/ml), and aprotinin (0.1%; Sigma Chemical Co.). For each 100-mg tumor, 500 μl of extraction buffer was added and tumors homogenized on ice (Tissue Tearor; Fisher Scientific, Pittsburgh, PA). Protein content of samples was determined by the Bradford method (Sigma Chemical Co.). Samples (20 μg/sample) were subjected to 15% SDS-PAGE under reducing conditions, after which proteins were transferred to nitrocellulose (BA-S 85; Schleicher and Schuell, Keene, NH) using standard methods (35). Blots were blocked then incubated in primary anti-VEGF mAb (Santa Cruz Biotechnology, Inc.; 5 μg/ml in Tween-20/Tris-buffered saline containing: 0.1% Tween 20 in Tris-buffered saline [100 mM Tris (pH 7.5) and 0.9% NaCl] for 1 h at room temperature. After washing, blots were sequentially exposed to biotinylated secondary antibody (30 min), avidin-biotin-HRP complex was added (Santa Cruz Biotechnology), then developed in luminol (Santa Cruz Biotechnology). Blots were then stripped (Immunopure IgG Elution Buffer; Pierce, Rockford, IL) and reprobed for actin expression (Santa Cruz Biotechnology).

VEGF and actin were quantified by densitometry using Un-Scan-It Automated Digitizing System software (Silk Scientific Corp., Orem, UT). Relative expression of VEGF was calculated as the ratio of VEGF:actin.

**Comparative RT-PCR for PlGF.** PlGF immunoblots were not quantifiable, and so comparative RT-PCR was used to semiquantify the relative abundance of PlGF mRNA in untreated, 3- and 5-week RAIT-treated LoVo and Calu-3 tumor samples (n = 3 tumors/time point). LoVo and Calu-3 were the
only tumors for which samples suitable for RNA extraction were available. To compare the relative abundance of PlGF mRNA after RAIT, total RNA was extracted from tissue stored at $-80^\circ\text{C}$ in 200-mg tumor tissue/ml of Tri-Reagent (Sigma Chemical Co.). RNA extractions and first-strand cDNA synthesis were done at the same time to insure similar extraction and synthesis conditions for all of the samples. First-strand cDNA was synthesized (oligodeoxythymidylic acid) from equal amounts of RNA for each tumor type (Thermoscript RT-PCR System; Life Technologies, Inc., Gaithersburg, MD). Comparative PCR (36–38) was performed in two phases. For the first phase, each individual sample of cDNA was amplified simultaneously for actin and PlGF using a low cycle number to insure linear amplification. $\beta$-actin was used as an internal PCR control for amount of cDNA in each reaction. Primers were designed from published sequence of human $\beta$-actin (GenBank XM 004814) and PlGF cDNA (GenBank NM 002632) to produce products of $\sim300$ and 200 bp, respectively. Primers: PlGF 5'$\text{ACCATCCAGCTCCTAAAGAT}$3' and 5'$\text{TA-GAAGCTTGCTAACCTCCGGGAACAGCCTCGCC}$3'  
actin 5'$\text{CCCGAGCGACAGGCTGCTTT}$3' and 5'$\text{CACACGCGCTTATTGAGAAG}$3'

A master mix was made consisting of Platinum Taq DNA polymerase (1 unit/reaction; Life Technologies, Inc.), buffer and 1.82 mM MgCl$_2$ and 200 $\mu$M deoxynucleotide triphosphates (supplied by manufacturer), and 1.82 mM MgCl$_2$ and sufficient water to make 50 $\mu$l final volume for each reaction. Actin or PlGF primers were then added (10 pmol of primer/reaction) to aliquoted master mix. These mixes were divided into reaction tubes, and equal amount of cDNA added to each. Amplification conditions were 94$^\circ$ for 30 s, 55$^\circ$ for 30 s, and 72$^\circ$ for 1 min for 24 cycles. Each reaction ($5\mu$l) was analyzed by electrophoresis in a 2% agarose gel and then photographed. Photographed bands were digitized using Un-Scan-It software (Silk Scientific, Inc.). Comparative amount of PlGF mRNA was expressed as the ratio of mean PlGF band density:mean actin band density for each time point. Mean actin signal was similar ($\pm10\%$) across all of the time points in LoVo, whereas the PlGF signal decreased. For Calu-3, mean actin signal was similar for untreated and 3-week samples but was only 38% of untreated at week 5. Despite the decrease in actin level in the week-5 Calu-3 samples, mean PlGF signal increased at week 3 and was 2.6-fold over untreated at week 5.

For the second phase of the analysis, all of the samples were reanalyzed using combined cDNAs (equal amounts of cDNA from each sample in a time point group) followed by PCR and analysis as described above. Results from combined samples were similar to those obtained when samples were analyzed separately. Results are reported in “Results” and fold-increase over untreated levels is shown (so a number of $<1$ in RAIT-treated samples indicates decreased relative PlGF mRNA abundance).

**Statistics.** Results are shown as mean $\pm$ SD or SE. IHC data were subjected to one-way (unpaired) ANOVA analysis. $P < 0.05$ was considered significant.

**RESULTS**

To document how RAIT alters underlying mechanisms of tumor revascularization, four human tumor xenografts were examined for treatment-induced changes in angiogenic factor expression up to 6 weeks after treatment. On the basis of biological clearance and physical decay, a single dose of RAIT delivers a continuous exponentially decreasing dose of radiation, so that after a single dose of $^{131}$I-IgG, $\sim80\%$ of the radiation dose is delivered to the tumor during the first week.
and the remaining 20% is delivered during week 2. Tumor growth arrest, measurable by the end of week 2, persists until weeks 4–6, when tumors often resume growth (26, 27). At the MTD, RAIT sensitivity as measured by growth inhibition is highest in GW-39 and LoVo. HT-29 and Calu-3 are more resistant to RAIT, show less growth inhibition than GW-39 and LoVo, and resume growth after treatment sooner (26, 27).

Treating at the MTD delivers different doses of radiation to the tumors over 2 weeks (cGy) depending on the tumor. However, treating with the MTD most closely simulates delivery of anti-tumor therapy in the clinic.

Angiogenic Growth Factor Expression in UTs and during Weeks 1 and 2 after RAIT. Angiogenic growth factors VEGF, ang-1, or ang-2 produced by tumor cells most likely bind receptors on endothelial cells or endothelial cell-associated pericytes to elicit vascular development in the tumor. Previous reports indicate that VEGF, ang-1, and ang-2 from human tumor cells bind and activate receptors on mouse endothelium in xenografts (39, 40). As a consequence, both tumor cell and perivascular growth factor signals were evaluated by IHC. Antibodies used were specific for both human and mouse growth factors.

At week 1, transitory but in most cases nonsignificant elevation of at least one angiogenic growth factor (VEGF, ang-1, or ang-2) could be detected in tumor cells by IHC in all of the tumors except Calu-3 (Fig. 1A). Tumor cell ang-1 was elevated 1.8-fold at week 1 in LoVo.

IHC measurement of most of the growth factors exhibited a biphasic pattern during weeks 1–2 (acute) when radioantibody is present versus weeks 2–6 during tumor growth arrest (recovery; Fig. 1A). For example, HT-29 VEGF rose during week 1 but had declined to untreated levels by week 2, after which expression increased and peaked at week 4 after RAIT. During weeks 1–2, VEGF, ang-1, or ang-2 staining on or near tumor blood vessels did not vary significantly from UT for the same time point (not shown).

Tie-1. By week 2 perivascular expression of orphan receptor Tie-1 increased in all four of the tumors 1.7–2-fold and remained elevated until week 4–5 (P < 0.05 in LoVo, Calu-3, and GW-39 at week 2; IHC).

Post-RAIT Changes in HT-29 (Weeks 2–6). Tumor cell VEGF increased significantly at week 4 (P < 0.001; IHC; Fig. 1A). VEGF staining of tumor blood vessels was elevated during weeks 3 and 4. Immunoblot analysis of whole tumor protein lysates also indicated a 1.6-fold relative increase in VEGF (relative to actin) at week 4 after RAIT (Fig. 1B).

Expression of VEGF receptor flk-1 on tumor vessels paralleled up-regulated VEGF, increasing 2.5-fold over UT controls at week 3 (Fig. 2; Fig. 3, A and B; P < 0.04). HT-29 was the only tumor to increase expression of flk-1 significantly after RAIT (Fig. 2). In addition, HT-29 was the only tumor to significantly increased staining for VEGF on or near blood vessels during weeks 2–6 (Fig. 3, C and D). The other VEGF receptor, flt-1, remained near UT levels until week 6 after RAIT. Early post-RAIT up-regulation of PlGF was also noted (weeks 1 and 2; P < 0.05; Fig. 4).

Endothelial cell proliferation and function are also controlled by another set of secreted growth factors, ang-1 and -2,
and their only known receptor, Tie-2. IHC indicated vascular Tie-2 was elevated between weeks 3–6 in HT-29, significantly at week 4 (P < 0.05; Fig. 2). In addition, increased Tie-2 ligand ang-2 was observed at week 4 (P < 0.02; Fig. 1A).

**Post-RAIT Changes in LoVo (Weeks 2–6).** Tumor cell VEGF in UT LoVo controls increased between weeks 1 and 6. This increase contrasts with the other tumor lines for which VEGF in UT samples remained almost static with time (Fig. 1A). VEGF levels in RAIT-treated LoVo were consistently at or below time-matched UT controls by both IHC and immunoblot (Figs. 1A; Fig. 3, E and F). ELISA of untreated LoVo, like IHC, indicated rising VEGF in UT samples with time (not shown). Expression of both VEGF receptors flk-1 and flt-1 on tumor vessels remained near UT levels (Fig. 2).

Because time or tumor size-dependent elevation of VEGF in UT LoVo samples was seen only in tumor cells and not on tumor vessels (IHC), we asked whether LoVo tumor cells might express VEGF receptors flk-1 or flt-1. VEGF receptor flt-1 was almost undetectable on tumor cells, but flk-1 was moderately expressed on UT tumor cells (not shown). However, after treatment, tumor cell flk-1 became undetectable. Flk-1 expression on UT LoVo tumor cells may indicate a role for VEGF/receptor interactions in tumor cell growth; RAIT treatment down-regulates both tumor cell VEGF and flk-1.

LoVo, because of reduced post-RAIT VEGF, was assayed for PlGF also (2, 3, 15–19, 41, 42). Neither RAIT-treated nor UT tumor cells developed a signal for PlGF at any time point (Fig. 3, I and J). Tumor vessel staining for PlGF was faint in UT tumors and did not change after RAIT (Fig. 3, I and J). PlGF mRNA was faintly detectable by comparative RT-PCR (see “Materials and Methods”); this analysis showed a relative decrease in PlGF mRNA abundance (compared with actin) at both weeks 3 and 5 after RAIT (41% of UT, week 3; 23%, week 5). Vascular Tie-2 increased and peaked at weeks 3 and 4 (week 4, P < 0.05; Fig. 2), although no significant increases were seen in tumor cell production of the growth factor ligands ang-1 and -2 by tumor cells. Perivascular staining for ang-1 was elevated during weeks 3 and 4 (not shown).

**Post-RAIT Changes in Calu-3 (Weeks 2–5).** Calu-3 IHC indicated decreased tumor cell VEGF during weeks 4 and 5 after RAIT (Fig. 1; Fig. 3, G and H). Immunoblot showed no change in VEGF expression at week 4 compared with untreated tumors (Fig. 1B). VEGF receptor flk-1 remained near UT level on tumor vessels during weeks 3–5 post-RAIT (Fig. 2). However, sustained but nonsignificant increase of flt-1 on tumor vessels was detected at weeks 2–5 (Fig. 2).

Because Calu-3 VEGF was reduced during weeks 3–5 when flt-1 was consistently elevated, tumor sections were assayed for PlGF by IHC and comparative RT-PCR. Untreated Calu-3 was faintly positive for tumor cell PlGF. However, after RAIT, tumor cell PlGF production increased during weeks 1–3 (P < 0.05; Fig. 3, K and L; Fig. 4). Moderate to heavy expression of PlGF was also detected on tumor vasculature (Fig. 3L). Comparative RT-PCR (see “Materials and Methods”) showed a 1.5- and 8-fold increase in PlGF mRNA expression over untreated samples at 3 and 5-weeks after RAIT, respectively. Calu-3 tumor PlGF may, by binding flt-1, transmit proliferative signals to endothelium during post-RAIT recovery.

**Post-RAIT Changes in GW-39 (Weeks 2–6).** As shown in Fig. 1A, VEGF expression was elevated after RAIT; immunoblot analysis also indicated an increase in VEGF at week 4 (Fig. 1B). IHC did not indicate a treatment-related increase in vessel-associated VEGF receptor flk-1 (Fig. 2). Tumor vessel flt-1 rose during weeks 1 (P < 0.05) and 2, and tumor cell and vascular PlGF peaked at week 3 (Fig. 4).

GW-39 tumor cell ang-1 remained at UT levels but ang-2 was significantly elevated at week 6. Tumor vessel Tie-2 peaked nonsignificantly at week 2 (1.8-fold both time points).

Table 1 summarizes significant (IHC) up-regulation of angiogenic growth factor and receptors during weeks 1–6 after RAIT. When growth factor/receptor pairs are taken together, HT-29 up-regulated ang-2/Tie-2 and VEGF/flk-1. PlGF/flt-1 were mobilized in Calu-3. LoVo up-regulated ang-1/Tie-2; and GW-39 did not significantly up-regulate any growth factor/receptor combinations investigated.

**Hypoxia.** To document changes in tumor hypoxia after RAIT and to relate these changes to induction of VEGF or PlGF expression, LoVo and Calu-3 tumor-bearing mice were injected with pimonidazole before tumor harvest (Ref. 34; see “Materials and Methods”). In all of the UT tumors, faint indication of hypoxia was mostly seen in areas adjacent to necrosis and comprised <20% of the viable portion of the tumor section. After RAIT, up to 50% of the apparent live tumor area was hypoxic, not only near necrosis, but also in areas distal to necrosis and in vascularized areas (Fig. 3, M, N, P, and Q: Calu-3 and LoVo, respectively).

VEGF and flt-1 expression are induced by hypoxia (43,
44), but hypoxia suppresses PIGF expression (15, 45–47). VEGF- and PI GF-stained sections of LoVo and Calu-3 were examined microscopically to determine whether hypoxic regions overlapped VEGF- or PI GF-positive areas at week 2 (48–50). VEGF signal overlapped hypoxic regions but was also seen in nonhypoxic areas in both treated and untreated samples in Calu-3 (not shown). Contrary to expression patterns reported for normal tissue (15), PI GF in RAIT-treated Calu-3 was observed in hypoxic regions as well as in nonhypoxic regions (Fig. 3O). These results suggest a dysregulation of PI GF expression in Calu-3 after RAIT.

DISCUSSION

In this study we used well-characterized human tumor xenograft models and treatment with RAIT to investigate which underlying mechanisms of revascularization might influence resistance or sensitivity to RAIT. In addition to killing tumor cells, RAIT most likely damages blood vessels, disrupting delivery of nutrients and O2 to tumor tissue (51). Therefore, recurrence of the tumor likely depends on rapid reestablishment of vessels for delivery of oxygen and nutrients to remaining live tumor cells. We hypothesized that regrowth would be preceded by post-treatment angiogenesis, which would manifest as increased expression of growth factors and growth factor receptors.

As in most clinical treatments, animals with similar tumors (e.g., colonic) were given an equitoxic dose of RAIT (MTD), although dose of radiation (cGy) actually delivered is tumor-specific. We expected that similar angiogenic responses would be observed in the three colonic tumors. However, we found that each of the four tumors responded to RAIT by unique changes in regulation of growth factor/receptor pairs. Differences in post-RAIT angiogenic potential between tumors arising from anatomically similar sites (e.g., colon carcinomas) may partly explain differences in treatment outcome. Ongoing studies will additionally explore the phenomena described here as a function of radiation dose to the tumor.

HT-29 and Calu-3, the most RAIT-resistant tumors, displayed several significant growth factor and receptor elevations, including endothelial cell mitogenic VEGF or PI GF; flk-1, flt-1 and Tie-2/ang-1 or ang-2. Activation of VEGF/flk-1 expression by HT-29 preceded ang-2/Tie-2 increase (weeks 4–6). This is in agreement with other reports showing that ang-2 is up-regulated by VEGF (52), and follows the pattern of angiogenesis shown during establishment of tumors (23). The coordinated induction by HT-29 of these early and late-phase angiogenic pathways post-RAIT may enhance revascularization and tumor survival, because ang-2/Tie-2 destabilizes endothelial contact with surrounding pericytes, and in concert with VEGF, augments endothelial proliferation (7). It is unknown if PI GF can substitute for VEGF in this interaction, but PI GF is also up-regulated in HT-29 after treatment and may also enhance post-treatment relapse by enhancing vascular recovery. Up-regulation of PI GF in recovering tumors, even when VEGF expression decreased as in Calu-3 suggests a heretofore unreported role for PI GF in tumor revascularization and recurrence. Additional studies are under way to delineate the role of PI GF in tumor recovery.

Analysis for VEGF using ELISA and whole tumor lysates showed no difference between untreated and treated for all of the tumor lines (not shown). Use of whole tumor lysates for ELISA may mask the local and specific changes in VEGF expression that can be detected using IHC. When the same lysates were used for immunoblot analysis, relative amount of VEGF increased in HT-29 and GW-39 in parallel with the IHC results.

In contrast to HT-29, the two tumor lines most sensitive to RAIT, GW-39 and LoVo, did not display a full response involving both endothelial growth factor production (VEGF/PI GF) and the mobilization of Tie-2 angiogenesis. The most RAIT-sensitive tumor, GW-39, slightly up-regulated VEGF in tumor cell cytoplasm (IHC), but neither ang-1 nor -2 increased before week 6. Increased PI GF/flt-1 might mediate some proliferation, but late-phase Tie-2 pathway elements remained unchanged until week 6 (ang-2). Asynchronous responses by either VEGF/PI GF or Tie-2-related angiogenic pathways may exacerbate susceptibility to RAIT by delaying revascularization. LoVo mobilized only ang-1/Tie-2, which possibly stabilized surviving vessels. However, without endothelial proliferative and differentiation signals from VEGF or PI GF, establishment of new microvasculature might be slow. In agreement with this hypothesis, the results of an additional study suggest that LoVo tumor vessel recovery begins after week 3 in contrast with HT-29, which shows significant recovery before week 3 (53).

VEGF overproduction observed in treated HT-29 and possible growth-related expression in UT LoVo contrasts with the relatively small amount of activity in Calu-3 and GW-39. The VEGF pathway, a common target of antiangiogenic therapy (12), may, therefore, have a minor role in the maintenance or recovery of some tumors.

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<th>Table 1 Summary table indicating significant up-regulation of growth factors and their receptors (IHC)</th>
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Tie-1 was up-regulated between weeks 2 and 5 in all four of the tumors (not shown). Although one study suggests that interference with the action of Tie-1 does not affect vascularization of newly forming tumors (23), the ability of this receptor to maintain endothelial cell integrity and influence microvessel network formation may be essential for post-treatment recovery, especially in tumors such as LoVo that exhibit relatively weak angiogenic growth factor mobilization.

Evidence that LoVo tumor cells may require autocrine VEGF-mediated signals for survival and growth is provided. VEGF-dependent tumor growth has been hypothesized for breast carcinoma and mesothelioma (54–57) but has not been reported for colon carcinomas. For these tumors, it is possible that treatment targeting VEGF or flk-1, besides inhibiting angiogenesis, might have direct antitumor effects.

Our previous work indicated that vascular permeability of Calu-3 increased and that of HT-29 decreased 2 weeks after RAIT (33, 58). VEGF mediates increased vascular permeability through flk-1-mediated signaling (59). VEGF and flk-1 levels at week 2 were investigated to determine whether increased expression paralleled increased permeability. Our results show VEGF at untreated levels in both tumors and flk-1 elevation in HT-29 only; so it seems unlikely that the increased permeability of Calu-3 results from increased VEGF, although basal levels of VEGF binding to flk-1 on endothelial cells or pericytes may account for permeability increase. Elevated PIGF may mediate increased vascular permeability in Calu-3 (60, 61). Permeability changes documented in the former study may result from another mechanism(s).

VEGF staining overlapped hypoxic regions of both LoVo and Calu-3 at week 2 after RAIT, although both tumors did not significantly up-regulate VEGF overall. These observations are in agreement with other reports, documenting up-regulation of VEGF during hypoxia (41, 42). Indications of PIGF in hypoxic regions of RAIT-treated tumors were not in keeping with many previous reports, which show that in normal trophoblasts and thyroid, hypoxia down-regulates PIGF. Evidence for hypoxic induction of PIGF during inflammation and in fibroblasts has been reported (19, 62). However, this is the first report that documents induction of PIGF during tumor recovery, with evidence that hypoxia may play a role in its expression. Although atopic expression of PIGF has been documented in primary human tumors (63), its role in tumor pathology has not been established. If PIGF is abnormally induced by tumor hypoxia or from treatment-induced stress, then its activity may enhance tumor relapse by stimulation of angiogenesis or by other heterofore uncharacterized mechanisms such as survival or growth enhancement of tumor cells, or by interaction with VEGF (42).

This study focused on surviving tumor cell up- or down-regulation of growth factors; however, stromal cells, especially vessel pericytes, may also be a source of angiogenic growth factors (64). Whether the angiogenic mechanisms highlighted here are preprogrammed responses to cytotoxic treatment or are RAIT-specific is currently under investigation. However, the results of this study do suggest that mobilization of multiple angiogenic growth factor/receptor systems after RAIT may confer resistance to treatment and promote recurrence. Angiogenic growth factors produced by tumor cells may enhance nonendothelial cell vessel mimicry reported in melanomas and reported recently in carcinomas (65). In addition, treatment protocols exploiting inhibition of more than one specific revascularization mechanism may be efficacious in preventing tumor recurrence and proliferation of metastases. Experimental results that establish the separate as well as the redundant functions of these and other factors in post-treatment recovery of tumor vessels will aid in development of therapeutic targets that will prevent recurrence of malignancies.

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