Discontinuous Schedule of Bevacizumab in Colorectal Cancer Induces Accelerated Tumor Growth and Phenotypic Changes

Abstract
Antiangiogenics administration in colorectal cancer patients seemed promising therapeutic approach. Inspite of early encouraging results, it however gave only modest clinical benefits. When AAG was administered with discontinuous schedule, the disease showed acceleration in certain cases. Though resistance to AAG has been extensively studied, it is not documented for discontinuous schedules. To simulate clinical situations, we subjected a patient-derived CRC subcutaneous xenograft in mice to three different protocols: 1) AAG (bevacizumab) treatment for 30 days (group A) (group B was the control), 2) bevacizumab treatment for 50 days (group C) and bevacizumab for 30 days and 20 without treatment (group D), and 3) bevacizumab treatment for 70 days (group E) and 70 days treatment with a drug-break period between day 30 and 50 (group F). The tumor growth was monitored, and at sacrifice, the vascularity of tumors was measured and the proangiogenic factors quantified. Tumor phenotype was studied by quantifying cancer stem cells. Interrupting bevacizumab during treatment accelerated tumor growth and revascularization. A significant increase of proangiogenic factors was observed when therapy was stopped. On withdrawal of bevacizumab, as also after the drug-break period, the plasmatic VEGF increased significantly. Similarly, a notable increase of CSCs after the withdrawal and drug-break period of bevacizumab was observed ($P < .01$). The present study indicates that bevacizumab treatment needs to be maintained because discontinuous schedules tend to trigger tumor regrowth, and increase tumor resistance and CSC heterogeneity.

Introduction
Angiogenesis is a fundamental event in tumor growth, progression, and metastasis. Vascular endothelial growth factor (VEGF), a potent angiogenic molecule, is associated with tumor progression and metastatic dissemination in several solid and hematopoietic malignancies [1]. The role played by VEGF has led to the development of therapeutic strategies that selectively target this pathway. Bevacizumab (Avastin), a humanized monoclonal anti-VEGF antibody, the first antiangiogenesis drug approved in combination with chemotherapy, was shown to prolong survival in patients with metastatic colorectal cancer (CRC) [2]. Nevertheless, after several years of anti-VEGF therapy in patients with solid tumors, the benefits are found to be less than satisfactory. Most patients, whatever their tumor types, ultimately exhibit resistance to VEGF-targeted therapy. Moreover, when anti-angiogenics (AAG) are administered on a discontinuous schedule or during drug-break periods (because of secondary

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transitory effects or choice of strategy), tumor regrowth has sometimes been observed [3–5]. Rapid tumor vasculature and regrowth have also been shown when AAG therapy was halted [6,7]. These observations could have clinical implications. In a continuous treatment program, it resulted in the prolongation of survival time inspite of tumor progression, as was noted in a clinical trial [8]. In clinical practice, AAG treatment may be temporarily halted or indefinitely abandoned. Though resistance to AAG has been extensively studied, it has not been documented in case of temporary interruption or definitive abandon.

The hypothesis therefore is that the neovascularized tumors can adapt to the presence of angiogenesis inhibitors, evade the therapeutic blockade imposed on angiogenesis [9–11], and develop resistance. The current experimental evidence suggests that there are at least four distinct adaptive mechanisms during evasion to AAG therapies: 1) increased pericyte coverage of the tumor vasculature, serving to support its integrity and attenuate the necessity for VEGF-mediated survival signaling; 2) activation and/or upregulation of alternative proangiogenic signaling pathways within the tumor; 3) recruitment of bone marrow–derived proangiogenic cells; and 4) activation and enhancement of invasion and metastasis to provide access to normal tissue vasculature without obligate neovascularization [12,13].

There are reports showing that inhibition of VEGF signaling in vitro and in vivo leads to compensatory increase in the expression of VEGF family ligands [12–15]. In view of these results, Yamagishi and collaborators postulated that the phenotypic changes induced by chronic inhibition of VEGF did not necessarily depend on compensatory pathways activated by VEGF family ligands but could most likely be attributed to other pathway(s) [16]. Many studies have similarly shown that loss of VEGF signaling in cancer cells, induced by either VEGF pathway targeting agents or VEGF gene disruption, enhances migration, invasion, and metastasis of tumor cells in vitro as well as in vivo [15–18]. Actually, we now know that other proangiogenic signaling pathways can stimulate blood vessel growth and promote blood vessel survival even when the VEGF pathway is blocked. Preclinical studies have identified numerous candidates involved including epidermal growth factor; EGF [19], fibroblast growth factor 1 and 2; FGF1 and FGF2 [9,20], hepatocyte growth factor; HGF [21], and placental growth factor; PIgf [22]. PIgf is a VEGF homolog. Clinical studies have shown that PIgf levels correlate with poor prognosis in various cancers including CCR and are also upregulated in cancer patients treated with VEGF inhibitors [23]. In the same way, HGF has also been identified in the vast majority of solid tumors [24] that are associated with an aggressive phenotype and poor prognosis [25]. It has also been demonstrated that hypoxia-inducible factor-1α (HIF-1α) plays an important role in the resistance to VEGF inhibition [26]. It has been proposed that the inhibition of VEGF-pathway could lead to increase of hypoxia, which in turn could induce a selection of tumor cells able to survive in low-oxygen environment [27]; it may also follow alternate compensatory proangiogenic pathways enabling persistent neovascularization [28].

Because of their self-renewal and multipotent properties, the current hypothesis is that cancer stem cells (CSCs) are another important source for recurrent tumor growth and accelerated disease progression. The increase of CSCs in response to hypoxia has first been reported in glioblastoma and breast cancer [29,30] and in colon cancer [31]. Based on experimental evidence, we can conceive broadly the mechanism behind evasive resistance, but little is known about the reversibility of these changes after cessation of anti-VEGF therapy.

In the current study, we demonstrate in a patient-derived (PDX) colon cancer xenograft mice model that a discontinuous schedule of bevacizumab effectively induces an acceleration of tumor growth, a rapid revascularization, and an upregulation of proangiogenic factors, but above all, it induces a modification of the heterogeneity of the CRC xenografts by significantly increasing the CSCs.

The present work endeavors to determine whether a withdrawal or a drug-break period actually accelerates the progression of colon cancer, and if so, it aims to understand the underlying mechanism(s).

**Materiel and Methods**

**Mice**

Female athymic mice (Swiss-nude), aged 4-5 weeks, were purchased from Charles River Laboratories (Saint Germain sur l’Arbresle, France). The mice were acclimatized for 1-2 weeks before tumor transplantation. Animal housing, handling, and all procedures involving mice were performed in accordance with the national animal care guidelines (European Commission directive 86/609/CEE; French decree no. 87-848).

**Patient-Derived Tumor Xenograft Models**

CR-IGR-0014P is a colon tumor of a human patient obtained from the Gustave Roussy Institute (Villejuif, France) at the initiative of the Oncodesign Consortium [32]. Tumor fragments were subcutaneously grafted into 6-week-old female nude mice, and the animals were sacrificed when tumors reached approximately a volume of 1000 mm³. We performed subcutaneous implantation of a 2-mm³ fragment in the neck area of experimental female nude mice with the tumors obtained from these sources.

**Treatments**

Anti-VEGF monoclonal antibody bevacizumab (Genentech BioOncology, South San Francisco, CA) was obtained from the pharmacy at Saint Louis Hospital (Paris, France). It was diluted in phosphate-buffered saline (PBS) 1× (CaCl₂ and MgCl₂ free), and 100 μl/mouse was injected intraperitoneally on a twice-a-week (Tuesday and Friday) schedule at 5 mg/kg. As control, 100 μl/mouse of PBS 1× (CaCl₂ and MgCl₂ free) was also injected intraperitoneally twice a week. As bevacizumab is photosensitive, it was stored in the dark at 4°C. The mice were randomized, and treatments started when the volume of tumors reached 100±30 mm³. The size of the tumor was assessed with a caliper (large diameter= D and small diameter= d) to obtain the volume V = D × (d/2)² twice a week.

The mice were subjected to three different treatment regimens, each regimen composing of two groups (Figure 1):

- AAG (bevacizumab) treatment for 30 days (group A). Group B was the control (n=6).
- bevacizumab treatment for 50 days (group C) and also bevacizumab for 30 days and 20 subsequent days without treatment (group D) (n=5).
- bevacizumab treatment for 70 days (group E) and a 70-day treatment with a drug-break period intervening between day 30 and 50 (group F) (n=5).

It was not possible to maintain a control for the 50- and 70-day regimen because of the long duration of the treatment, resulting in volume of the tumor exceeding the limits set by the strict rules of ethics.
Figure 1. Treatment regimen. The mice were treated with three different regimens to analyze the effect of a discontinuous therapy with bevacizumab. The first regimen (D30) was composed of six mice per group. Group A was treated for 30 days with bevacizumab versus the nontreated control, group B. The second regimen (D50) was composed of five mice per group. Group C was treated for 50 days continuously with bevacizumab versus group D treated for 30 days and 20 subsequent days without treatment. The third regimen (D70) was composed of five mice per group. Group E was treated for 70 days continuously with bevacizumab versus group F treated for 70 days with a drug-break period from day 30 to day 50.

Radioimmunology
We collected peripheral blood samples in heparinized tubes by cardiac puncture from anesthetized mice (immediately before the mice were sacrificed). Blood was then centrifuged at 2000 rpm during 15 minutes and the plasma collected. Radioimmunometry assays were performed on plasma for VEGF, PlGF, and HGF according to the manufacturer’s instructions (R&D Systems Inc., France).

Enzyme-Linked Immunosorbent Assay
Enzyme-linked immunosorbent assay (ELISA) was performed on tumor homogenates with VEGF, PlGF, and HIF-1α and on plasma for SDF-1α according to the manufacturer’s protocol (R&D Systems Inc., France).

Immunofluorescence Staining
The identification of endothelial cells and pericytes of tumor vessels was performed by double immunofluorescence staining. Tumors were OCT embedded and frozen. Frozen specimen were cut into 30-μm sections and were fixed on glass slides for 15 minutes in 4% paraformaldehyde in PBS. Endothelial cells were labeled with rat anti-CD31 (BD Biosciences, France, 1/50), and pericytes were labeled with rabbit anti-desmin (Thermo Scientific, France, 1/200). Immunofluorescence detection was done using secondary antibodies Alexa Fluor 488-labeled goat anti-rat IgG for anti-CD31 and Alexa Fluor 594-labeled goat anti-rabbit IgG for anti-desmin (Life Technologies, France, 1/400).

Tissue sections were examined with an Observer Z1 (Zeiss) fluorescence microscope. For endothelial cells and pericytes, the ratio of CD31 or Desmin-stained area to total area was calculated.

Solid Tumor Disaggregation
Colon CSC was prepared by generating a single-cell suspension from human colon cancer tissues. Tumor tissues were finely cut and minced with scalpel in 5 ml of stem cell medium. The tissue suspension was further disaggregated by vigorous pipetting. Enzymatic disaggregation was achieved by incubating the tissues for 2 hours at 37°C with collagenase II (1.5 mg/ml) and DNase I type II (600 U/ml)(Sigma Aldrich, France). Passage through a Ficoll column removed necrotic cells, debris, and red blood cells. The population of mononuclear cells obtained was then resuspended in 5 ml of stem cell medium and serially filtered by using sterile gauze 100-μm, 70-μm, and 40-μm nylon meshes.

Flow Cytometry and Cell-Sorting EXPERIMENTS
Quantification of cancer stem cells. To minimize experimental variability and loss of cell viability, all experiments were performed on fresh tumor cell suspensions prepared before flow cytometry. Antibody staining was performed in PBS 1× containing 1% bovine serum albumin and 2% fetal bovine serum. Analysis of ALDH enzymatic activity was performed using the Aldefluor system (StemCell Technologies Inc., France) according to the manufacturer’s instructions. The cells were subsequently washed and stained with antibodies. Antibodies used in this study included: anti-human CD44-Pacific Blue (Exbio, Clinisciences, France), anti-human CD133-APC (Miltenyi Biotec, France) and anti-human CD166-PE (Beckman Coulter, France). Flow cytometry analysis was performed by using a MoFlo Astrios cell sorter (Beckman Coulter, France).

Quantification of Endothelial Progenitor Cells. Blood samples were freshly collected in heparinized tubes by cardiac puncture from anesthetized mice (immediately before mice were sacrificed). Following lysis of red blood cells, the peripheral blood mononuclear cells were incubated for 30 minutes at 4°C with the following antibodies: anti-mouse VEGFR-2-FITC antibody, anti-mouse CD34-PE antibody, anti-mouse anti-CD45.1-FITC antibody, or immunoglobulin G isotype as controls (eBiosciences, Paris, France). The cells were then examined using a BD Biosciences LSRII flow cytometer (BD Biosciences, Le Pont de Claix, France). The percentage of positive cells in each sample was calculated using Kaharu flow cytometry analysis software (Beckman Coulter France S.A.S, Villepinte, France).

Statistical Analysis
Results were subjected to statistical analysis using GraphPad Prism v6.0 software (GraphPad, San Diego, CA). The results are expressed as mean±SEM. Student t test was applied for the analysis of surface immunofluorescence staining. Statistical significance was determined by using Mann-Whitney U test. All statistical tests were two-sided, as the comparison was always done between group A versus group B, group C versus group D, and group E versus group F. The P values <.05 were considered to be statistically significant.

Results
Kinetics of Tumor Growth
The kinetics of tumor growth was followed for the three regimens of bevacizumab treatment. For the first regimen, that is, mice receiving 30 days of bevacizumab treatment (group A), we observed a significant delay in tumor growth (P<.01) compared to the nontreated animals (Figure 2A). In the second regimen, the discontinuation of bevacizumab (group D) resulted in an acceleration of tumor growth after day 40, with a significance value (P<.05) at day 50 (Figure 2B), and could be considered as a rebound effect. In the last regimen, after a drug-break period (group F) in a long-term treatment (70 days), we did not observe any significant difference compared to the continuous 70-day treatment (group E) (Figure 2C). Taken together, these results indicate that 1) a continuous treatment
bevacizumab effectively induces a delay in tumor growth and 2) on the other hand, when bevacizumab was administered on a discontinuous schedule, an accelerated tumor regrowth emerged.

**Tumor Vascularization**

The regression and regrowth of tumor vessels were studied by immunofluorescence staining of endothelial cells (using CD31 as marker) and pericytes (using desmin as marker). The CD31-positive endothelial cells in blood vessels within the tumors were visibly reduced after 30 days of bevacizumab treatment, group A (Figure 3A) compared to group B (Figure 3B). After withdrawal of treatment (group D) in the 50-day regimen, there was a conspicuous increase in tumor vessels displaying both types of cells, endothelial and pericytes. It showed abundant desmin-positive cells completely enveloping the CD31-positive cells (Figure 3D) as compared to group C (Figure 3C).

In the 70-day regimen (group E), the desmin-positive cells were more abundant than the CD31-positive cells (Figure 3E). However, in group F, there was a clear increase in CD31-positive cells (Figure 3F).

After quantification, the regression of tumor vessels caused by VEGF blockade was observed in every group (A, C, and E) receiving continuous treatment (Table 1). In all interrupted groups (D and F), the tumor vessel network was more prominent, with an increase in CD31-positive cells (group D, +78% versus group C, \( P < 0.01 \)) and (group F, +35% versus group E, \( P < 0.05 \)).

The effect of bevacizumab-mediated blockade of VEGF on the pericyte population was only observable after the first 30 days. After interruption of treatment (group D), a rebound phenomenon was observed with a major increase in the pericycle surface area (+57% versus group C; \( P < 0.05 \)). However, in the prolonged and uninterrupted treatment (group E), contrary to expectations, the pericyte population increased (+46% versus group F; \( P < 0.01 \)). The increase in pericytes under these circumstances may be indicative of a takeover of tumor revascularization.

**Quantification of VEGF, HIF-1\( \alpha \), and Other Proangiogenic Factors**

We examined the influence of bevacizumab in our colon human tumor xenograft-bearing model for inducing molecular changes during and after cessation of treatment. We therefore assayed VEGF and PlGF concentrations in tumor and in plasma for the different treatment regimens. In addition, HGF and HIF-1\( \alpha \) were also assayed in plasma and tumor, respectively.

In our model, treatments with bevacizumab downregulates the VEGF concentration in tumor tissues and in plasma (group A) \( (P < 0.05 \) and \( P < 0.01 \), respectively) (Figure 4, A and C). The reduction in VEGF concentration remained valid even after a prolonged treatment (groups C and E). It was also associated with a similar reduction in HGF (groups A, C, and E) (Figure 4E). We noticed no difference between the groups for the tumor PlGF levels; however, there was a significant difference between groups C and E (Figure 4B). In case of all interrupted treatments (groups D and F), the different components such as VEGF, plasmatic PlGF, and HGF increased dramatically \( (P < 0.01 \). The plasmatic VEGF concentration increased in case of rebound tumor growth (group D) by 81% \( (79.8\pm6.1 \text{ vs. } 14.7\pm2.9 \text{ pg/ml}; P < 0.01 \) (Figure 4C). In group F (drug-break period group), the increase was 88% \( (130.7\pm18.3 \text{ vs. } 14.9\pm3.2 \text{ pg/ml}; P < 0.01 \).
Figure 3. Fluorescence microscopic images of tumors stained for CD31 (green; endothelial cells) and desmin (red; pericytes). The staining with CD31 (A) showed a regression after bevacizumab for 30 days compared to the nontreated tumors; (B) there are no obvious differences in expression of desmin in the two groups. (C) When treatment was maintained for 50 days, the vascularization was still held in check. (D) But when the treatment was stopped, the tumors became highly revascularized by CD31 and desmin-positive cells. (E) With a prolonged bevacizumab treatment of 70 days, the pericytes (desmin-positive cells) are alone able to revascularize the tumors. (F) In group F a second round of treatment does not prevent revascularization by CD31-positive cells. Scale bar (applies to all images): 100 μm.
**Table 1. Measurements Showing the Surface of CD31-Positive Cells and Desmin-Positive Cells in Blood Vessels in the Three Different Regimens**

| Regimen | Surface Expression | Group A | Group B | P       |
|---------|--------------------|---------|---------|---------|
|         | Surface CD31/total surface (%) | 1.83±1.84 | 5.14±4.8 | <.01    |
|         | Surface desmin/total surface (%) | 1.25±1.36 | 1.50±1.49 | NS      |

| Regimen | Surface expression | Group C | Group D | P       |
|---------|--------------------|---------|---------|---------|
|         | Surface CD31/total surface (%) | 1.15±1.13 | 5.42±5.8 | <.01    |
|         | Surface desmin/total surface (%) | 4.32±2.97 | 10.15±8.32 | <.05    |

**Endothelial Progenitors**

We quantified circulating EPCs by flow-cytometry and assayed in plasma the stromal derived factor 1α (SDF-1α), its downstream effector [33], by ELISA. We observed no significant differences in EPCs and SDF-1α in the three regimens of treatment of our mice model (Figure 5, A and B).

**Cancer Stem Cells**

We examined whether our three different regimens were able to bring about phenotypic changes by increasing colon CSCs population. We checked subpopulation markers with ALDH1⁺, CD44⁺, CD166⁺ and with ALDH1⁺, CD44⁺, CD166⁺, CD133⁺. In the 30-day treatment regimen, the use of bevacizumab did not change the level of CSCs in tumors. In the groups with interruption of bevacizumab treatment, we observed, for the two subpopulations, an increase in CSCs (group D and F) (Figure 5, C and D). In the case of ALDH1⁺, CD44⁺, CD166⁺, CD133⁺ cells, the tumor CSC levels increased dramatically (P<.01) with a 57% increase in group D and a highly significant increase of 90% (7.99±5.1 versus 0.67±0.34 cells %; P<.01) for group F (Figure 5D). It was clear that the interruption of bevacizumab treatment affected the size of CSC population in tumors.

**Discussion**

The VEGF antagonist bevacizumab is of interest in treatment of CRC and is largely used by clinicians worldwide. There was a great optimism that inhibition of the VEGF pathway would represent an effective AAG therapy. VEGF pathway-targeted drugs have shown initial clinical benefits; however, in the vast majority of patients, the disease was ultimately found to progress and was therefore disappointing in the long run.

In practice, treatment may be subjected to interruption and then to a restart (drug-break period) or to abandon en route. In these cases, patient benefits should be clearly evaluated. Clinical observations in some patients treated with AAG revealed rapid regrowth and revascularization during the drug-break period or drug withdrawal [4,7]. The therapeutic and mechanistic relevance of these regrowth and revascularization is still unclear.

The reason we chose the PDX model, compared to the human CRC cell line–derived xenograft model, is that the former is more representative of the heterogeneity of human cancers in terms of clinical parameters, histopathology, molecular pattern, and sensitivity to drugs [32].

This study focuses on the effects of a discontinuous or drug-break period of VEGF inhibition and uses a PDX CRC mouse model for the purpose. First, we followed the kinetics of tumor growth. The 30-day regimen confirmed the efficiency of bevacizumab in inhibiting tumor growth. Our results showed that stopping VEGF inhibition (50-day regimen) led to a rapid and significant regrowth as observed by Cacheux et al. Our findings are consistent with the notion of a rebound following interruption of angiogenesis inhibition. Cacheux et al. [4] have also reported that bevacizumab reinduced a tumor response on resumption of treatment of their patients. We have not been able to confirm their claims however interesting.

We also examined the tumor revascularization in the three anti-VEGF regimens; the goal was 1) to determine the rate of blood vessel regrowth in tumors after removal of VEGF inhibition and 2) the role of endothelial cells and pericytes in this revascularization. First, we noticed that an anti-VEGF treatment without interruption always prevented the formation of new tumor vessels originating from CD31-positive cells. After withdrawal of bevacizumab (50-day regimen), a conspicuous increase in the number of tumor blood vessels, composed of endothelial cells and pericytes, was observed. Also, an abundant number of desmin-positive cells were found to completely cover the CD31-positive cell population. This strongly suggests that the withdrawal of VEGF inhibition induces the acceleration of tumor blood vessel regrowth. The study of Mancuso et al. [6] has addressed the issue of revascularization on spontaneous pancreatic islet cell carcinomas in RIP-Tag2 mice treated for 7 days with an inhibitor of VEGFR. They found that the withdrawal of treatment ultimately led to full vascularization of implanted tumors.

CD31-positive cells were significantly lower in the continuous bevacizumab treatment group (70 days) compared to the drug-break group. Comitantly, the reduction in CD31-positive cells led to a significant increase in desmin-positive cells (pericytes), indicating that perhaps they are able to function in a compensatory manner and take over the revascularization of tumors. However, after a drug-break
period, we observed a significant decrease of desmin-positive cells on one hand and a simultaneous increase in CD31-positive cells, indicating that a second round of bevacizumab did not prevent a revascularization and “tumor escape.” Surprisingly, Mancuso et al. [6] found that a second round of anti-VEGFR reduced tumor vascularity as much as the first round, which was in contradiction with the results obtained by us which may be due to differences in the protocol and experimental design adopted by the two groups.

Indeed, in our case, the longer duration of treatment may have been the cause for triggering a higher level of resistance. The differences in response to treatment may also be due to the difference in the type of tumors: human colon versus murine pancreatic carcinomas, or it could come from the difference in the drugs used: we used anti-VEGF, while Mancuso et al. [6] used an anti-VEGFR.

The increase in VEGF in the 50-day regimen after halt in treatment (group D) correlated well with the recruitment of endothelial CD31-positive cells. There was also an increase in VEGF in the plasma compared to group C. Even though no difference was found for the intratumor VEGF in the 50-day regimen, there was a remarkable increase of VEGF in the plasma as well as within the tumor in case of the 70-day regimen. This observation supports well the finding that the regrowth tumor vasculature involves endothelial cells in group F. It is clear that much of the regrown tumor blood vessels originated from bevacizumab-resistant cells.

This is the first study that examines the effect of a long and protracted treatment by bevacizumab. We can conclude that, in the two cases (noninterrupted treatment versus drug-break period), a clear resistance to bevacizumab was observed. It is likely that when VEGF was inhibited by bevacizumab, the signal responsible for endothelial cell activation “switched off,” and consequently the pericytes took over and revascularized the tumors. We know that VEGF has no effect on pericytes recruitment, but we also know that pericytes participate in the maturation of blood vessels [34]. The presence of pericytes is a sign of vessel maturation and therefore is certainly the consequence of resistance to the antiangiogenics. Our findings indicate that tumor blood vessels undergo rapid regression when VEGF is inhibited. Our results also underline the importance of pericytes, besides CD31-positive cells, as potential targets in cancer therapy. A very recent study showed that after discontinuation of anti-VEGF, the hepatic revascularization exhibited hyperpermeability and profound structural changes that permitted tumor cell intravasation and extravasation and therefore promoted cancer metastasis [35].

Figure 4. Discontinuation of bevacizumab induces activation of alternative proangiogenic pathways. In order to understand the mechanisms of evasive resistance, including revascularization as a result of upregulation of alternative proangiogenic signals, we analyzed molecular changes of VEGF, PlGF, HGF, and HIF-1α in our three regimens. (A and B) Intratumor assay by ELISA. The concentration is expressed in pg/mg of proteins (pg/mg protein). (A) VEGF. (B) PlGF. (C, D and E) Plasma assay by radioimmunology. The concentration is expressed in ng/ml. (C) VEGF. (D) PlGF. (E) HGF. (F) HIF-1α; also intratumor assay by ELISA expressed in pg/mg protein. Data are presented as mean±SEM. .01<*P<.05; .001<**P<.01.
This highlights the importance of maintaining AAGs or targeting both endothelial and pericytes cells. There are ongoing clinical trials or trials in development that aim to target simultaneously endothelial cells and pericytes and assess the potential benefits for efficient antitumor therapy.

Besides VEGF, we also turned our attention to two other proangiogenics factors, namely, PlGF and HGF. We studied PlGF from intratumoral regions and from plasma, and HGF from plasma. Again, the three different regimens of treatment, as in the case of VEGF, were employed in order to determine whether bevacizumab was able to induce discernable changes in the behavior of PlGF and HGF. The PlGF assay in intratumor indicated no difference among the three regimens. However, a significant difference between group C and group E was seen, indicating that the duration of bevacizumab exposure had an obvious effect in the activation of these proangiogenics growth factors. In the group with discontinuation of treatment (group D), the plasma assay for HGF and PlGF showed a significant upregulation as also in the 70-day regimen after a drug-break period (group F). Such compensatory pathway activation after an AAG treatment has been already documented by several authors [9,16]. The present study has revealed that a discontinuous, as compared to the continuous, schedule of bevacizumab induced an increase in proangiogenic factors including PlGF and HGF involved in the development of resistance.

Hypoxia is one of the major conditions in which HIF-1α, partly through SDF-1α, is involved in the recruitment of bone marrow-derived cells such as EPCs for tumor growth [33]. These EPCs have the capacity to differentiate into mature endothelial cells and to participate in the formation of new tumor blood vessels [40,41]. We presume that circulating EPCs may be involved in the rebound tumor growth observed in our PDX colon cancer model. However, to our surprise, we noticed no significant differences in the circulating EPC levels between the different groups in our three regimens. Apparently, the EPCs seem not to be involved in the acceleration of tumor revascularization after discontinuation of bevacizumab.

The CSCs have been isolated from a variety of tumor types, including CRC. Most anticancer therapies are believed not to target CSCs, and therefore, these cells are spared and survive even after drug administration. These CSCs may play a role in cancer recurrence [42]. The recurrent tumor growth, observed in patients treated with AAG, raised the question as to whether there exists a subpopulation of cells in colon cancer responsible for the resistance to AAG. A preclinical study has shown that by inducing hypoxia, the...
antiangiogenesis therapies also increased the ALDH+ CSC in human breast cancer xenografts [30]. A report has described that the CD133+CSC population in colon cancer was resistant to bevacizu-
mab [31], which further supports that these cells survive the drug
treatment and therefore could be a source of repopulation of the
tumor.

Using a panel of markers that are known to be present on CSCs of
human CRC [43], we quantified by flow cytometry two types of CSC
subpopulations in the three regimens. We demonstrate here that a
discontinuous schedule of bevacizumab modifies the expression
profile of CRC xenografts by significantly increasing CSCs. In view of
these findings, we may consider other mechanisms that may intervene
in evasive resistance through stimulation and enrichment of the two
types of subpopulations. Studying the level of HIF-1α may provide
further information.

A significant decrease in HIF-1α between groups A and C as also
between groups A and E was noticed. This may be due to the fact
that, after initial AAG treatment, the intratumor hypoxia increases
in the first regimen (30 days). In the 50-day and 70-day regimen, the
increase in proangiogenic factors (VEGF, PIGF, and HGF) on one
hand and the tumor revascularization on the other may explain the
decrease in HIF-1α. The increase in the proangiogenic factors, in case of
discontinuous schedules (group D and group F), raised the
question about the hypoxia-mediated stimulation of CSCs.

There are in fact preclinical and clinical trials that test whether
HIF-1α blockade could increase the therapeutic benefits of inhibitors
of VEGF signaling. The association of an AAG and topotecan (a
topoisoerase I inhibitor that blocks the accumulation of HIF-1α) has
already been tested on a glioblastoma [28] and on an ovarian
腫瘤 cell animal models [44]. In both cases, they have been found to
display an increased antitumor activity. Unfortunately, the study in
which the topotecan has been tested in combination with bevacizumab in patients with refractory solid tumors was halted
prematurely [45].

We have seen from our studies that, in both cases (uninterrupted
versus interrupted treatment), the tumors may show resistance to
AAG, but the discontinuous schedule is the worst-case scenario. A
recent paper described a specific clinical situation named maintenance
strategy on a metastatic CRC strategy [46]. Our results certainly
recent paper described a specific clinical situation named maintenance
AAG, but the discontinuous schedule is the worst-case scenario. A

Conflict of Interest

The authors declare no conflict of interest.

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islet tumors. *Cancer Cell* 8, 299–309.

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blacked could increase the therapeutic benefits of inhibitors of
VEGF signaling. The association of an AAG and topotecan (a
topoisoerase I inhibitor that blocks the accumulation of HIF-1α) is
already been tested on a glioblastoma [28] and on an ovarian
cancer animal models [44]. In both cases, they have been found to
display an increased antitumor activity. Unfortunately, the study in
which the topotecan has been tested in combination with bevacizumab in patients with refractory solid tumors was halted
prematurely [45].

We have seen from our studies that, in both cases (uninterrupted
versus interrupted treatment), the tumors may show resistance to
AAG, but the discontinuous schedule is the worst-case scenario. A
recent paper described a specific clinical situation named maintenance
strategy on a metastatic CRC strategy [46]. Our results certainly
throw some light on the events involved and attempt to provide an
explanation. Clinical experience provides proof-of-principle that
AAG therapy is a valid therapeutic approach, but the full potential
of this strategy remains yet to be completely exploited.

Finally, this study has emphasized the fact that a discontinued
schedule of bevacizumab modifies the tumor cell heterogeneity as a
result of rebound and repopulation. Any successful future develop-
ment of AAG therapy will require further understanding of how this
tumor heterogeneity occurs and how to target it effectively. It is a key
goal not just for AAG therapy but for all cancer therapeutics. Our
findings showing that a discontinuous schedule of bevacizumab led to
an increase in CSCs provide a potential explanation for the limited
clinical benefits from AAG therapies as of today. Improving the
clinical efficacy of AAG treatments will require developing therapeutics that target these resistant CSC population.

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