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

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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.

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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 definitely 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; PlGF [22]. PlGF is a VEGF homolog. Clinical studies have shown that PlGF 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 mm^3. We performed subcutaneous implantation of a 2-mm^3 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× ($CaCl_2$ and $MgCl_2$ 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× ($CaCl_2$ and $MgCl_2$ 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 mm^3. The size of the tumor was assessed with a caliper (large diameter=D and small diameter=d) to obtain the volume $V=D \times (d)$ [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.

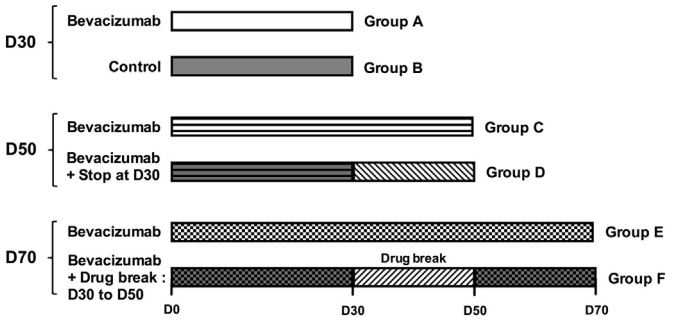

Figure 1. Treatment regimen. The mice were treated with three different regimens to analyze the effect of a discontinuous therapy with bevacizumab. The first regimen (D30) was composed of six mice per group. Group A was treated 30 days with bevacizumab versus the nontreated control, group B. The second 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 anaesthetized 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 anaesthetized 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 Kaluza 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 means±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 2*A*). 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 2*B*), 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 2*C*). Taken together, these results indicate that 1) a continuous treatment

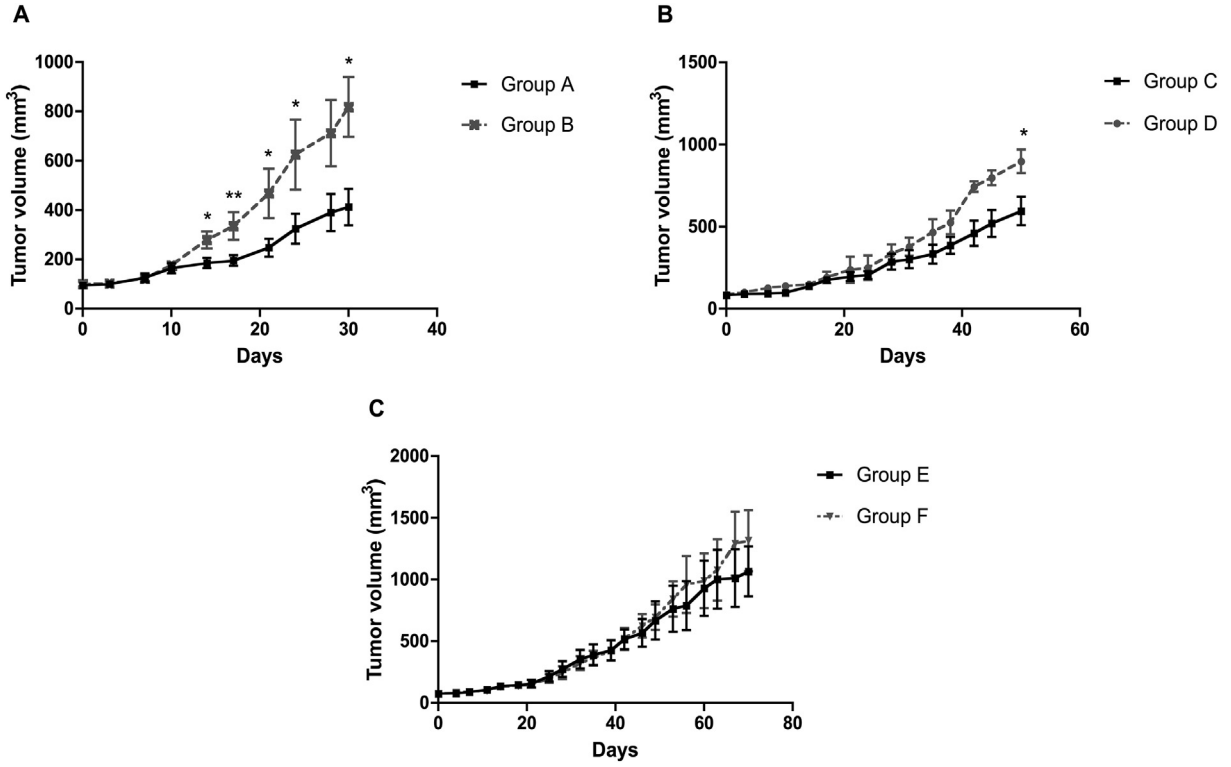

Figure 2. Kinetics of tumor growth on colon PDX tumor treated with a discontinuous schedule of bevacizumab. (A) Bevacizumab administered 30 days (group A) induced a significant delay in tumor growth compared to nontreated control (group B). (B) Bevacizumab discontinued at day 30 (group D) induced a rapid resumption of tumor growth as compared to group C. (C) When bevacizumab was stopped at day 30 (group F), there was a slight but notable resumption of tumor growth which was not restored after rechallenge at day 50 compared to group E, treated for 70 days uninterrupted. Data are presented as mean±SEM. .01<*P<.05; .001<**P<.01.

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 3*A*) compared to group B (Figure 3*B*). 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 3*D*) as compared to group C (Figure 3*C*). In the 70-day regimen (group E), the desmin-positive cells were more abundant than the CD31-positive cells (Figure 3*E*). However, in group F, there was a clear increase in CD31-positive cells (Figure 3*F*).

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<.01) and (group F, +35% versus group E, P<.05), respectively.

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 pericyte surface area (+57% versus group C; P<.05). However, in the prolonged and noninterrupted treatment (group E), contrary to expectations, the pericyte population increased (+46% versus group F; P<.01). The increase in pericytes under these circumstances may be indicative of a takeover of tumor revascularization.

Quantification of VEGF, HIF-1α, 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-1α 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<.05 and P<.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 4*E*). We noticed no difference between the groups for the tumor PlGF levels; however, there was a significant difference between groups C and E (Figure 4*B*). In case of all interrupted treatments (groups D and F), the different components such as VEGF, plasmatic PlGF, and HGF increased dramatically (P<.01). The plasmatic VEGF concentration increased in case of rebound tumor growth (group D) by 81% (79.8±6.1 versus 14.7±2.9 pg/ml; P<.01) (Figure 4*C*). In group F (drug-break period group), the increase was 88% (130.7±18.3 versus 14.9±3.2 pg/ml;

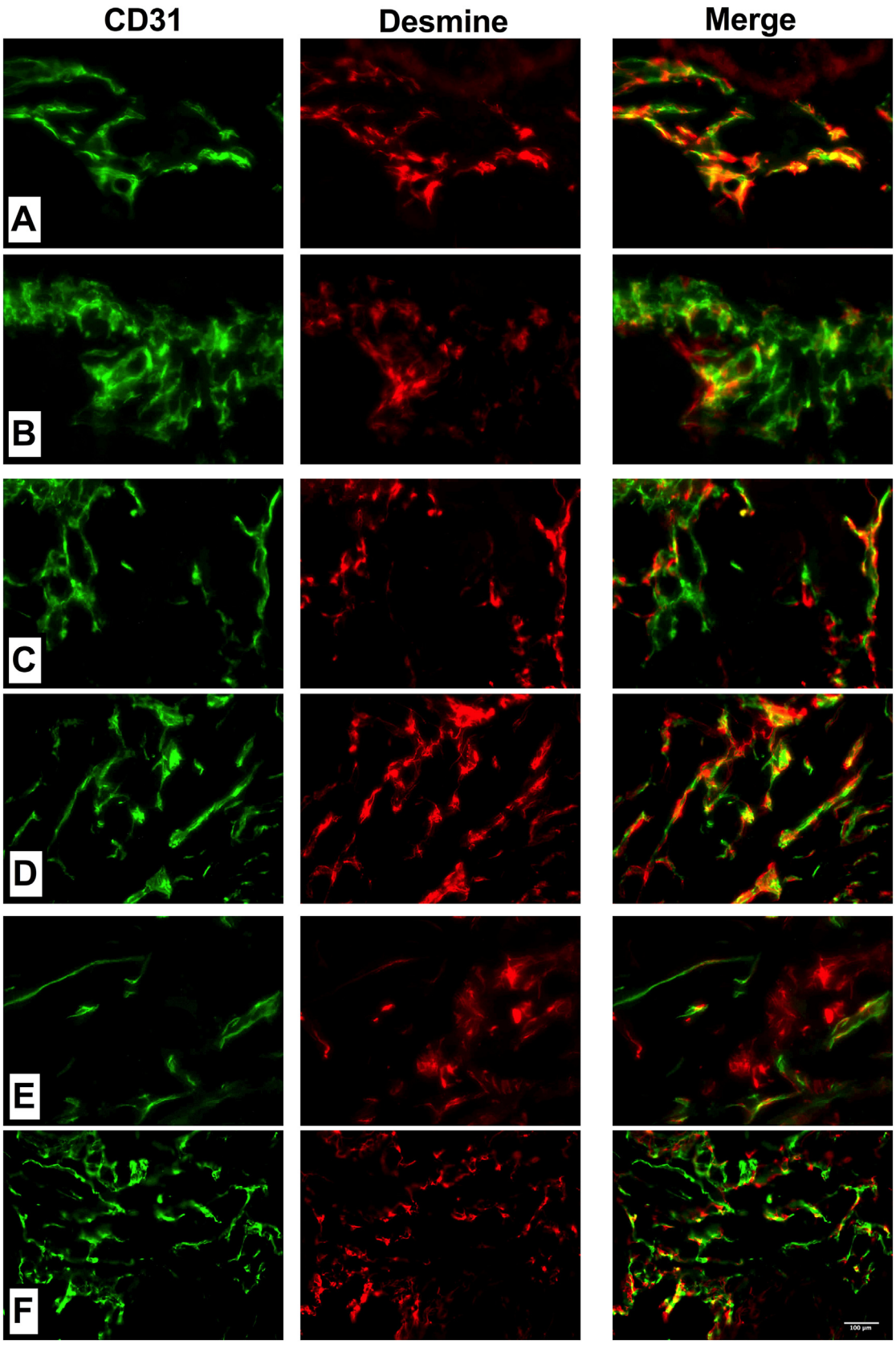

Figure 3. Fluorescence microscopic images of tumors stained for CD31 (green; endothelial cells) and desmin (red; pericytes). The staining with CD31 (A) showed a regression after bevacizumab for 30 days compared to the nontreated tumors; (B) there are no obvious differences in expression of desmin in 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

D30			
Surface Expression	Group A	Group B	*P*
Surface CD31/total surface (%)	1.83±1.84	5.14±4.8	*P*<.01
Surface desmin/total surface (%)	1.25±1.36	1.50±1.49	NS
D50			
Surface expression	Group C	Group D	*P*
Surface CD31/total surface (%)	1.15±1.13	5.42±5.8	*P*<.01
Surface desmin/total surface (%)	4.32±2.97	10.15±8.32	*P*<.05
D70			
Surface expression	Group E	Group F	*P*
Surface CD31/total surface (%)	1.14±1.22	1.77±1.0	*P*<.05
Surface desmin/total surface (%)	4.23±2.12	2.27±1.45	*P*<.01

In the 30-day regimen, there is a significant decrease of vascularity in group A, confirming the efficacy of this antiangiogenic to prevent the formation of new tumor vessels and at the same time eliminate existing tumor vessels. In the 50-day regimen, after withdrawal of bevacizumab (group D), vascular density increased significantly with significant increase of CD31 and desmin-positive cells. It indicates an acceleration of the tumor regrowth. In the 70-day regimen, there is also a significant increase of CD31-positive cells (group F), indicating that, after a second round of bevacizumab, the regrown tumor vessels are resistant to the antiangiogenics. Instead, in the noninterrupted group, the number of desmin-positive cells increased significantly (group E), indicating their ability to take over in place of CD31 in the tumor revascularization. Data are presented as mean±SEM. *P* values <.05 were considered to be statistically significant.

P<.01). Moreover, the VEGF concentration in group F was found significantly increased as compared to group D (Figure 4*C*). These results indicate that, in a second round of treatment, the tumors were able to escape from further effect of bevacizumab exposure.

The effect of the discontinuous schedule of bevacizumab-mediated VEGF inhibition on hypoxia was examined by assaying tumor content of HIF-1α by ELISA. A significant difference was observed only for the 70-day regimen, with a 57% decrease of HIF-1α in the group F (drug-break period) compared to the group E (70-day continuous treatment). The HIF-1α concentration for the group F was 526.6±219.2 versus 1241.6±86.5 pg/mg of proteins for group E (*P*<.05). Contrary to an expected increase of HIF-1α, we observed in fact a significant decrease of this protein in all the three groups treated without interruption (A, C, and E). The decrease obtained between group A and group C was 46%, while that between A and E was 51% (Figure 4*F*). These results are interesting in that the decrease of HIF-1α and the increase in proangiogenic factors (VEGF, PlGF, and HGF) seem to be interlinked.

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 5*D*). 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. Concomitantly, 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

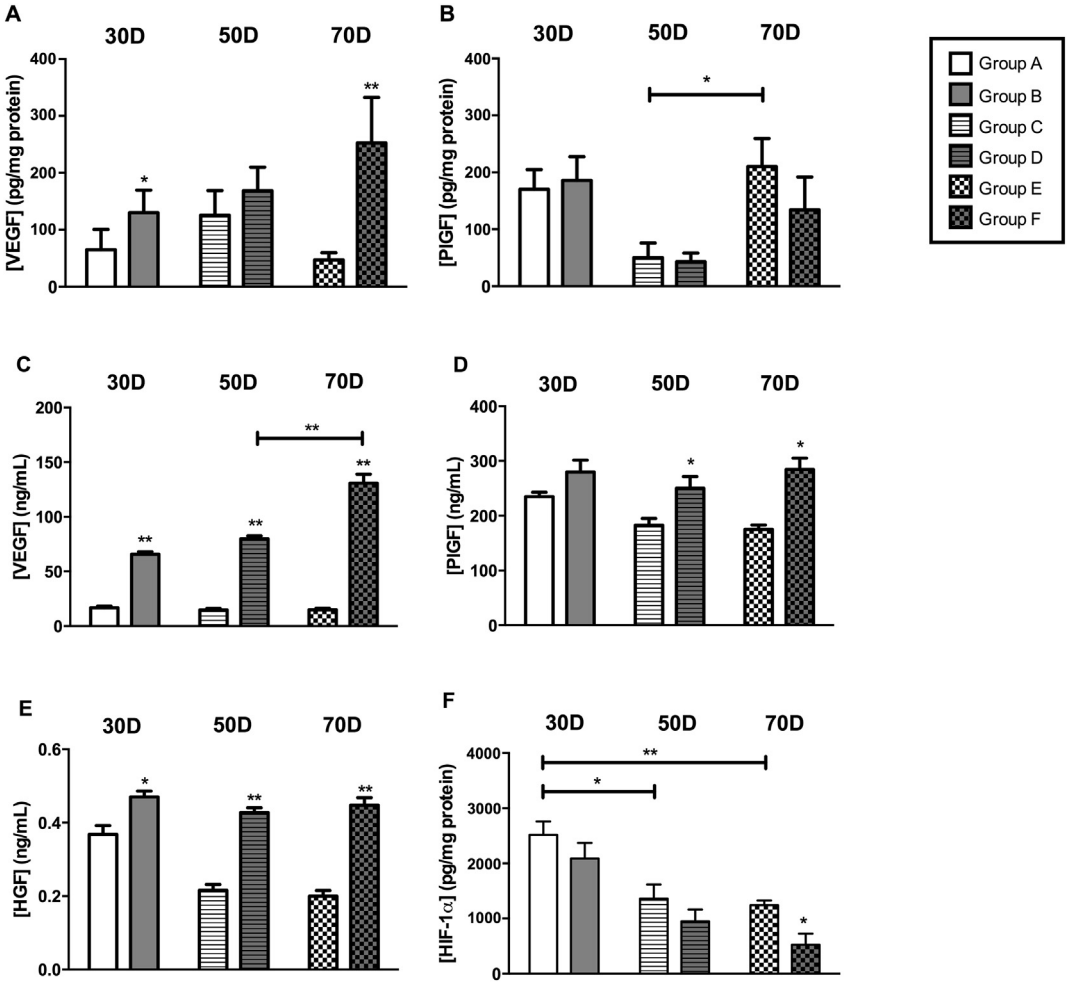

Figure 4. Discontinuation of bevacizumab induces activation of alternative proangiogenic pathways. In order to understand the mechanisms of evasive resistance, including revascularization as a result of upregulation of alternative proangiogenic signals, we analyzed molecular changes of VEGF, PlGF, HGF, 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.

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].

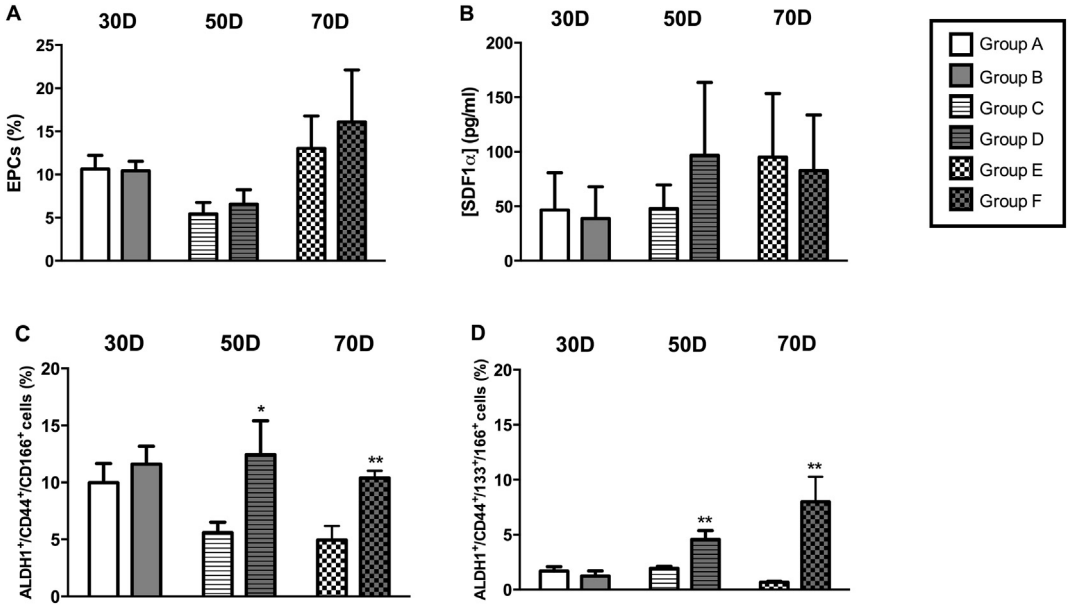

Figure 5. Discontinuous bevacizumab treatments do not modify EPC recruitment by SDF-1α but induces enrichment of CSCs. (A) The quantification by flow cytometry shows no differences between the groups in EPCs recruitments. (B) The SDF-1α assay also showed no difference between these groups. The assay concentration is expressed in pg/ml. We quantified, by flow cytometry, two types of CSC subpopulations, with ALDH1$^+$, CD44$^+$, CD166$^+$ and ALDH1$^+$, CD44$^+$, CD166$^+$, CD133$^+$cell markers, respectively. (C) In the first regimen, there was no difference between the different groups, but when bevacizumab was stopped (group D), there was a significant increase of CSCs, and the same result was observed after a drug-break period (group F) with a significant increase compared to the group E. (D) As for the first subpopulation, there was no difference between the different groups for the D30 regimen. After withdrawal of bevacizumab (group D), there was a significant increase of the CSCs as in the case of the drug-break period (group F). 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.

However, the concept that the activation and/or upregulation of alternative proangiogenic signaling pathways mediate resistance to AAG therapy is yet to be clinically validated. The majority of tyrosine kinase inhibitors used to treat patients (including brivanib, cediranib, dovitinib, sunitinib, sorafenib, vatalanib, and many others) are multitarget in nature and can suppress the signaling of several proangiogenic pathways. The diseases such as glioblastoma [36], hepatocellular carcinoma [37], and metastatic renal cell carcinoma [38] have been shown to progress inspite of treatment with these agents. This contrasts with the preclinical studies demonstrating a role for alternative signaling pathways and questions the relevance of alternative proangiogenic growth factors in mediating resistance to AAG therapy in patients [39]. In case one encounters resistance to AAG, it would be interesting, in a second round of treatment, to test another proangiogenic target. It should ultimately permit in establishing an effective therapeutic strategy that could be tested in the clinics.

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 bevacizumab [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 intratumoral hypoxia increases in the first regimen (30 days). In the 50-day and 70-day regimen, the increase in proangiogenic factors (VEGF, PlGF, and HGF) on one hand and the tumoral 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 topoisomerase I inhibitor that blocks the accumulation of HIF-1α) has 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 that underlines the importance of nonstopping AAG treatment 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 development 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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Conflict of Interest

The authors declare no conflict of interest.

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