Akt-Activated Endothelium Constitutes the Niche for Residual Disease and Resistance to Bevacizumab in Ovarian Cancer

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Abstract

Ovarian cancer is the second leading cause of cancer-related death in women worldwide. Despite optimal cytoreduction and adequate adjuvant therapies, initial tumor response is often followed by relapse suggesting the existence of a tumor niche. Targeted therapies have been evaluated in ovarian cancer to overcome resistant disease. Among them, antiangiogenic therapies inhibit new blood vessel growth, induce endothelial cell apoptosis, and block the incorporation of hematopoietic and endothelial precursor cells into new blood vessels. Despite in vitro and in vivo successes, antivascular therapy with bevacizumab targeting VEGF-A has limited efficacy in ovarian cancer. The precise molecular mechanisms underlying clinical resistance to anti-VEGF therapies are not yet well understood. Among them, tumor and stromal heterogeneity might determine the treatment outcomes. The present study investigates whether abnormalities in the tumor endothelium may contribute to treatment resistance to bevacizumab and promote a residual microscopic disease. Here, we showed that ovarian cancer cells activate Akt phosphorylation in endothelial cells inducing resistance to bevacizumab leading to an autocrine loop based on FGF2 secretion. Altogether, our results point out the role of an activated endothelium in the resistance to bevacizumab and in the constitution of a niche for a residual disease. Mol Cancer Ther; 13(12); 3123–36. ©2014 AACR.

Introduction

Neoangiogenesis is a primordial step for tumor growth and metastasis and its targeting is under investigation in many solid tumors (1–4). Human cancer cells express elevated levels of proangiogenic factors (5). Among them, Elevated expression of circulating VEGF-A is associated with poor prognosis in metastatic colorectal, lung, and renal cancer (6). Subsequently, the first antiangiogenic molecule developed to impair neangiogenesis targeted VEGF. Bevacizumab (Avastin, Genentech Inc.) is a humanized monoclonal antibody directed against all isoforms of VEGF-A (8). Bevacizumab prevents the binding of VEGF-A to its receptor VEGF-R1 (Flt-1) and R2 (Flt-2 or KDR). Although the function of VEGF-R2 in tumor angiogenesis has been characterized thoroughly, the function of VEGF-R1 is not yet well defined (9). Bevacizumab has been found clinically active in many solid tumors, such as colon, non–small cell lung, metastatic renal cell carcinoma, or glioblastoma (10–13). Currently, the U.S. Food and Drug Administration approves its use in combination with chemotherapy/immunotherapy in colon, lung, and renal cancers and in glioblastoma as a single agent. The mechanisms of action of bevacizumab are not perfectly understood. It could act by sequestering VEGF-A in the blood and/or tumor interstitium or induce normalization of VEGF-A concentration in these compartments (14). Despite initial efficacy, the withdrawal of bevacizumab can be associated to rapid tumor regrowth or a “rebound” phenomenon with accelerated clinical decline in patients (15, 16). Moreover, in case of tumor progression under bevacizumab, salvage chemotherapy has been quite inefficient, suggesting the emergence of a treatment-resistant phenotype (17). Most tumors will develop resistance to bevacizumab on a long term (18). While vessels were perceived as passively conducting nutrients to tumor cells, recent reports underline the perfusion-independent role of the endothelium. In such context, endothelial cells participate to an active...
cross-talk with tumor cells, resulting in the modification of their phenotype (19). Endothelial cells isolated from various tumors acquired genotype alterations as aneuploidy or abnormal multiple chromosomes (20) or displayed an activated profile through PI3K/Akt or MAPK pathway (21).

Ovarian cancer usually presents as locally advanced disease with involvement of the peritoneal cavity, and the use of bevacizumab is under investigation in several clinical trials. Here we hypothesized that the cross-talk between cancer and endothelial cells would induce an activated endothelial cell profile responsible for bevacizumab resistance and the constitution of a vascular niche. We demonstrated that the cross-talk between ovarian cancer cells (OCC) and established human umbilical vein endothelial cell line (HUVEC) resulted in an activation of Akt pathway among others. To model tumor endothelium, we used the Akt-activated endothelial cells (E4 ECs) to study the involvement of the endothelial component in the resistance to antiangiogenic therapy (22). We demonstrated that in the setting of tumor-endothelial cross-talk, endothelial cell activation of PI3K/Akt pathway induces an autocrine loop through the proangiogenic factor FGF2, bypassing the VEGFR pathway. We demonstrated that blocking FGF2 would efficiently reverse the resistance to bevacizumab. These results propose the use of stroma-directed synthetic therapy to overcome resistance in ovarian cancer.

Materials and Methods

Reagents and chemicals

Bevacizumab (trade name Avastin, Genentech) or 100 mg/16.6 mL paclitaxel (Ebetaxel, EBEWE Pharma) were obtained from National Center for Cancer Care and Research (NCCCR; Doha, Qatar) pharmacy. The bevacizumab doses used for patients’ treatment (5–15 mg/kg) correlates to a mean plasma concentrations value between 0.1 and 0.5 mg/mL; we diluted bevacizumab in serum-free medium to obtain final concentrations of 0.5, 1.0, 1.5, 2, and 2.5 mg/mL. Ly294002 (L9908), FGF receptor (FGFR) inhibitors PD17034 (P2499) and SU-5402 (SML0443) were purchased from Sigma-Aldrich and resuspended in DMSO.

Immunohistochemistry

Immunohistochemistry (IHC) was performed at the Segal Cancer Centre Research Pathology Facility (Jewish General Hospital, Montreal, Quebec, Canada). Tissue samples were cut at 4 μm, placed on Superfrost Plus stain slides (Fisher), and dried overnight at 37°C. The slides were then loaded onto the Discovery XT Autostainer (Ventana Medical System). All solutions used for automated IHC were from Ventana Medical System unless otherwise specified. Immunostaining for phospho-Akt was performed using a heat protocol. Briefly, rabbit monoclonal anti-phospho-Akt (Ser473; D9E, Cell Signaling Technology) diluted 1:50 in antibody diluent solution was manually applied for 30 minutes and then followed by the appropriate detection kit (Omnimap anti-Rabbit HRP). Slides were counterstained with hematoxylin for 4 minutes, blued with Bluing Reagent for 4 minutes, removed from the autostainer, washed in warm soapy water, dehydrated through graded alcohols, cleared in xylene, and mounted with Permount. Sections were analyzed by conventional light microscopy.

Cell culture

Established HUVEC were purchased and grown as recommended by ATCC. E4 ECs were obtained as previously described (22). Briefly, isolated HUVECs were transduced with the early 4 region (E4) of the adenoviral vectors (AdE4+) to generate durable PEC feeders. HUVECs and E4 ECs were cultured in M199 medium supplemented with 20% FBS, 1% penicillin/streptomycin, 0.1% heparin, and 0.1% endothelial cell growth supplement (ECGS) and maintained at 37°C in a humidified 5% CO2 atmosphere. HUVECs were seeded on plates coated with 0.1% gelatin and allowed to grow. Treatments were performed in serum growth factor-free medium over 24 hours. Human ovarian microvascular endothelial cells (HOMEt) were purchased from ScienCell research Laboratories (#7300) and grown according to the manufacturer’s instructions. Ovarian cancer cell lines SKOV-3 (HTB-77) and OVCAR-3 (HTB-161) were purchased from ATCC. Cells were grown in DMEM high glucose (Hyclone, Thermo Scientific) supplemented with 10% FBS (Hyclone, Thermo Scientific), 1% penicillin/streptomycin/amphotericin B solution (Sigma), 2 mmol/L L-glutamine (Sigma), 1× nonessential amino acids (Hyclone, Thermo Scientific). All cell lines were obtained between 2011 and 2013 from ATCC or ScienCell research laboratory. Upon receipt, cells were expanded and aliquots of cells at passage number <10 were stored frozen in liquid nitrogen. Cells from one aliquot were kept in culture for less than 2 months. The authors did not authenticate identity of each cell line purchased. GOC-2 and GOC-A2 are primary cell lines that were derived by Dr. Gotlieb’s laboratory. GOC-2 cells were isolated from a papillary serous ovarian cancer obtained after 4 courses of neoadjuvant chemotherapy with carboplatin/taxol and GOC-A2 from a stage IIIc serous ovarian cancer.

Cell proliferation assay

Cellular viability and proliferation were analyzed by both trypan blue exclusion and tetrazolium dye assay. HUVECs and E4 ECs cells were allowed to grow to 80% confluence in 96-well plates. Cells were then incubated with the different concentrations of bevacizumab in serum, cytokine-free medium for 24 hours and subjected to XTT assay as previously described by the manufacturer (TACS XTT cell proliferation assay kit, Trevigen, Inc.). Optical density was measured at 540 nm. Alternatively, treated cells were then collected and diluted in trypan blue dye (Sigma) for manual counting with hemocytometer.
Wound-healing and tube formation assays
Cells were grown to confluency in starvation medium (serum growth factor–free). Using a pipette tip, cells were scraped from the culture dish leaving a void space. Cells were washed with PBS and then incubated for 36 hours in the presence of the treatment. After incubation, wound closure was imaged by phase contrast microscope and analyzed using ImageJ software. Five random fields per well were examined. For the tube formation assay, 24-well culture plates were coated with Matrigel according to the manufacturer’s instructions. HUVECs and E4+ECs were seeded on coated plates at 5 × 10^4 cells per well in serum growth factor–free medium, treated and incubated at 37°C for 8 hours. Cells were then analyzed for tube formation with an inverted light microscope. The criteria taken into account were the branching point number being formed. Five random fields per well were examined. Cells seeded on coated plate in complete medium served as positive control.

Generation of a treatment-resistant HUVEC line
A treatment-refractory HUVEC line was developed by continuous exposure to bevacizumab. Briefly, HUVECs were

Figure 1. Akt pathway is activated in vivo and in vitro in ovarian cancer endothelium. A, representative IHC staining of phospho-Akt (Ser473) and CD31 in papillary serous adenocarcinomas specimens. Sections exhibit intense CD31 immunostaining colocalizing with pAkt staining (arrows) 20 ×. B, coculture with established OCC lines and isolated primary OCCs induced Akt activation in HUVECs. HUVECs cocultured for 24 hours with ovarian cancer cell lines were stained for phospho-flow cytometric analysis targeting Akt (S473) phosphorylation. A representative experiment depicting phosphorylation of Akt at S473 after coculture with OCCs is shown. The percentage of cells scoring positive for pAkt-Ser473 (red area) was determined by comparing cocultured HUVECs with their control (non-cocultured HUVECs). ***, P < 0.001.
Figure 2. Akt-activated endothelial cells (E4+ ECs) display resistance to bevacizumab. A, schematic representation of the experimental design used. B, effect of bevacizumab on the viability of E4+ ECs; their control HUVECs and HUVECs incubated with SKOV-3 conditioned media (CM) after 24-hour treatment with bevacizumab. Growth inhibition was assessed by XTT assay and normalized to the control (nontreated cells). C, E4+ ECs and HUVECs monolayers were incubated for 24 hours in serum and growth factor–free medium and treated with bevacizumab (2 mg/mL). The number of cells was visualized by phase-contrast microscopy (4×; scale bar, 500 μm). Viability was evaluated by flow cytometry using Live/Dead kit. Live cells are represented in green. D, effect of bevacizumab on cleavage of PARP in HUVECs, E4+ ECs, and HUVECs incubated with SKOV-3 CM. (Continued on the following page.)
were exposed to stepwise increases in bevacizumab concentrations. The initial bevacizumab exposure was at a concentration of 0.1 mg/mL. After cells had regained their exponential growth rate, bevacizumab concentration was doubled; this procedure was repeated until selection of a clone resistant to 1 mg/mL of bevacizumab. The resulting bevacizumab-refractory cell line was subcultured every 5 days and treated every 15 days with 1 mg/mL of bevacizumab to maintain a high level of antiangiogenesis resistance.

**Sphere formation assay**

E4 EC-eGFP and SKOV-3 were dissociated into single-cell suspension by trypsinization and further sieving through 40-μm cell strainers. E4 EC-eGFP and SKOV-3 cells (ratio 10:1) were then resuspended in 3-dimensional media consisting of DMEM/F12 supplemented with 2% B27, 20 ng/mL EGF, 20 ng/mL bFGF, and 5 μg/mL insulin in ultralow attachment plates. Primary cancer spheroids and angiospheres (SKOV-3:E4 EC-eGFP, ratio 1:10) started to form at day 3 and maintained up to day 7. Primary spheres were dissociated into single-cell suspension and replated as mentioned above to form secondary spheres. Seven-day-old secondary spheres were used for inducing tertiary spheres. Sphere formation was evaluated by ImageJ64 software based on the area of the sphere covered by SKOV-3. To evaluate E4 EC-eGFP survival under bevacizumab treatment, the area of the sphere covered by E4 EC-eGFP was majored.

**Analysis of viability and apoptosis detection by flow cytometry**

Viability was assessed by flow cytometry evaluation of Calcein AM staining as described by the manufacturer (Live Dead Viability/Cytotoxicity Kit, Molecular Probes, Invitrogen). Cells treated with bevacizumab (1.5 mg/mL), were harvested after 24 hours. Analyses were performed on SORP FACS Aria2 (BD Biosciences). Calcein AM fluorescence was acquired with 488 nm blue laser and 525/50 nm emission, ethidium homodimer was acquired with 640 nm red laser and 670/40 nm emission. Cytotoxicity was assessed by flow cytometric evaluation of Annexin V, propidium iodide (PI) staining cells as described by the manufacturer (FITC Annexin V Apoptosis Detection Kit I, BD Pharmingen). Cells were treated overnight with varying concentrations of Bevacizumab (0.1, 1, 1.5, 2, 2.5 mg/mL) and/or Ly294002 (10 μmol/L). PI and Annexin V were added and cells incubated at room temperature for 15 minutes. Stained cells were analyzed on SORP FACSAria2 using excitation at 535 nm and emission at 582/15 nm for PI and 488 nm excitation, 525/50 nm emission for Annexin-FITC. Data were processed with FACS-Diva 6.3 (BD Biosciences). Doubles were excluded by FSC-W × FSC-H and SSC-W × SSC-H analysis, single-stained channels were used for compensation, and fluorophore minus one (FMO) controls were used for gating (23).

**Phospho-flow analysis and intracellular detection**

After treatment or coculture, cells were fixed using BD Cytofix Fixation Buffer (BD Biosciences), followed by permeabilization at 4°C with BD PhosFlow Perm Buffer III (BD Biosciences). Conjugated antibodies were added to the cells for 45 minutes at 4°C. Phospho-Akt S473 phycocerythrin (PE)-conjugated antibody (BD Biosciences) and VEGF-A PE-conjugated antibody (R&D Systems) were acquired with 535 nm green laser and 582/15 nm emission. Phospho-STAT-3 (Y705) Alexa Fluor 647 (BD Biosciences) was acquired with 640 nm red laser and 670/14 nm emission. A total of 50,000 events were acquired per sample. Single-stained channels were used for compensation and FMO controls were used for gating.

**Western blot analyses, antibodies, and immunoprecipitation**

Immunoblot analysis were performed using the following antibodies from Cell Signaling Technology: phospho-Akt (S473) (#9271), Akt isoform sampler kit (#9940), FGF-2 (#3196), PARP (#9532), cleaved PARP (#9541), phospho-FGFR1 (#3472), phosphoSTAT3 (Y705) (#9145), phospho-STAT3 (S727) (#9134), STAT3 (#4904), phospho-ERK1/2 (#9101), phospho-Src (Y416) (#2101), actin (#3700); and VEGF-A from Santa Cruz Biotechnology (#SC-152). Immune reactive bands were visualized by using a developing solution (Western Lightening, Perkin Elmer LAS, NEL104). For immunoprecipitation, equal amounts of cell lysate were preabsorbed with 50 μL of protein A/G Plus agarose (Santa Cruz, Sc-2003) and then incubated overnight at 4°C with anti-Akt1, anti-Akt2, and anti-Akt3 antibodies. Immune complexes were precipitated with 50 μL of Protein A/G Plus agarose and then washed 3 times with lysis buffer. Immunoprecipitated proteins were eluted in Laemmli sample buffer and subjected to Western blot analysis.

**RT-PCR analysis**

Total RNA was isolated by TRIzol reagent (Invitrogen). Briefly, addition of chloroform, after the
centrifugation, separates the solution into aqueous and organic phases. RNA remains only in the aqueous phase. After transferring the aqueous phase, RNA is recovered by precipitation with isopropyl alcohol. A quantitative analysis of RNA was performed using a 2-step reverse transcription-quantitative PCR (GoTag 2-Step qRT-PCR System qRT-PCR, Promega) protocol according to the manufacturer's instructions. cDNA was synthesized from 2 μg total RNA, using the GoScript Reverse Transcription System and amplified using the GoTag qPCR Master Mix. Cycling conditions were 95°C for 2 minutes, 40 cycles of 95°C for 15 seconds, 60°C for 1 minute, 72°C for 10 seconds, 72°C for 5 minutes, and cooled to 4°C. The final extension step was continued for 5 minutes. Variation in cDNA loading was normalized to GAPDH expression, and relative expression values were determined using ΔΔCq method of relative quantification. Primer sequences are listed as Supplementary Table and were purchased from IDT.

Neutralizing anti-FGF2 assay

A monoclonal neutralizing mouse anti-FGF2 antibody (Millipore, clone bFM-1) was used for FGF2 neutralization at 1 and 5 μg/ml with or without bevacizumab. Cell viability was determined by XTT assay after 2 days of neutralization. E4 ECs stimulated with 20 ng/ml of FGF2 were used as positive controls. Isotypic IgG control was used as internal control, and serum-free medium as negative control.

FGF2 and VEGF-A secretion

We used a Human VEGF-A Quantikine ELISA Kit (DVE00, R&D Systems) and Human FGF-2 Quantikine ELISA Kit (DFB50, R&D Systems). Plates were read at 450 nm. Concentrations were interpolated from a standard curve.

Statistical analysis

All quantitative data were expressed as mean ± SEM. Statistical analysis was performed, using SigmaPlot 11 (Systat Software Inc.). A Shapiro–Wilk normality test, with a P = 0.05 rejection value, was used to test normal distribution of data prior further analysis. All pairwise multiple comparisons were performed by one-way ANOVA followed by Sidak–Holm post hoc tests for data with normal distribution or by Kruskal–Wallis analysis of variance on ranks followed by Tukey Post Hoc tests, in case of failed normality test. Paired comparisons were performed by Student t tests or by Mann–Whitney rank-sum tests in case of unequal variance or failed normality test. Statistical significance was accepted for P < 0.05 (*), P < 0.01 (**), or P < 0.001 (***)

Results

Cancer cell cross-talk with endothelial cells induces prosurvival signaling

Tumor endothelial heterogeneity and in particular endothelial Akt activation has been previously reported (24). Ovarian tumor vessels displayed heterogenous phospho-Akt staining (Fig. 1A). To investigate the role of soluble factors in tumor–endothelial cell communication, we established a Transwell coculture system of HUVECs and OCCs. We used (i) established OCCs lines (OVCAR-3, SKOV-3) and (ii) 2 primary OCCs isolated from papillary serous ovarian cancers (GOC-2 and GOC-A2). As shown in Fig. 1B, we observed heterogeneous Akt phosphorylation in HUVECs. OVCAR-3 cells induced less Akt phosphorylation than the other cell lines tested. SKOV-3, which are highly resistant to chemotherapy and have metastatic features, induced the higher level of Akt activation. We confirmed the “activated” phenotype in HUVEC by Western blotting (Supplementary Fig. S1).

E4 ECs display resistance to bevacizumab

We hypothesize that endothelium activation might play a role in bevacizumab resistance. The use of serum and cytokines to maintain HUVECs in culture might hinder cell-autonomous effect. Thus, to model a tumor-associated endothelium, we used our recently generated endothelial cell line E4 ECs that can survive in serum cytokine-
A

Phospho-Akt (S473)

pAkt (S473) quantification (normalized to control)

B

Absorbance (fold decrease)

C

T = 0

T = 36 h

Branching point number

E

Absorbance (fold decrease)

E4+EC control
E4+EC + Beva
E4+EC + Anti-FGF2
E4+EC + Anti-FGF2 + Beva

Ctrl
Anti-FGF2
Anti-FGF2 + Beva

Guerrouahen et al.

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free media (22) to study further mechanisms of resistance to bevacizumab. We first verified the presence of VEGF-A and VEGFR (mRNA and protein levels) in HUVECs and E4ECs (Supplementary Fig. S2A and S2B). We conducted several functional assays with E4ECs, their control HUVECs and HUVECs cocultured with OCCs (Fig. 2A). We evaluated the cell viability by XTT cell proliferation assay and FACS, after bevacizumab treatment. Doses of bevacizumab between 1 and 2.5 mg/mL did not significantly affect the number of viable E4ECs contrary to HUVECs at 24 and 48 hours (Fig. 2B and C and data not shown). Addition of VEGF-A (10, 25, 50 ng/mL) could not completely protect HUVEC from the decrease of viability (Supplementary Fig. S3A, top). Interestingly, HUVECs pretreated with VEGF-A before treatment with bevacizumab displayed higher survival (Supplementary Fig. S3A, bottom). We could illustrate the absence of an apoptotic phenomenon in E4ECs and HUVECs cocultured with SKOV-3 conditioned medium by the absence of cleaved PARP upon bevacizumab treatment (Fig. 2D). Besides the prosurvival signaling, VEGF-A also induces endothelial cell migration and vessel formation (25). Under serum growth factor-free conditions, we were not able to evaluate the motility of HUVEC in the presence or absence of VEGF-A (10, 25, 50 ng/mL) or bevacizumab treatment. But we observed that in E4ECs or HUVECs cocultured with SKOV-3 condition medium, bevacizumab was not able to prevent wound closure (Fig. 2E and Supplementary Fig. S3B and S3C).

In a tube formation assay, while HUVECs treated by bevacizumab alone or supplemented with VEGF-A resulted in unramified tube-like structures with dead ends, E4ECs or HUVECs cocultured with SKOV-3 condition medium maintained the capacity to form assembled capillary-like structures (Fig. 2F and Supplementary Fig. S3D and S3E). Concordantly, E4ECs displayed the same profile of resistance as that of HUVECs cocultured with SKOV-3 supernatants, confirming the use of E4ECs as a model. Interestingly, we also showed that bevacizumab had a profound effect on HOMECs compared with E4ECs (Supplementary Fig. S3F).

**Inhibition of Akt-mediated survival pathways increases bevacizumab effect**

We first showed that Akt phosphorylation was abrogated as early as 10 minutes after treatment with the pan-P13K inhibitor LY294002 (10 μmol/L; Fig. 3A and Supplementary Fig. S4A). LY294002 treatment alone did not decrease E4EC viability, but cotreatment with bevacizumab reversed E4EC resistance to antiangiogenic therapies (Fig. 3B and Supplementary Fig. S4B). This underlines the central role of Akt pathway in the resistance to bevacizumab treatment. To illustrate the essential role of Akt, we developed a bevacizumab-refractory HUVEC line. Treatment of this cell line with Ly294002 decreased Akt phosphorylation and restored the sensitivity to bevacizumab (Supplementary Fig. S4C). Western blot analysis and immunoprecipitation showed that Akt1 is the predominant isofrom expressed in E4ECs as well as the main form phosphorylated (Supplementary Fig. S4D). Additional angiogenic factors such as FGF2 (26) can compensate the blockade of VEGF (27). Interestingly, transcriptomic and RT-PCR analysis showed that FGFR1 mRNA was increased by 2.5-fold in E4ECs compared with HUVECs (Supplementary Fig. S4E). In all OCCs used in this study, while bevacizumab efficiently neutralizes VEGF-A, the expression of FGF2 could still be detected (Supplementary Fig. S4F). We observe that E4EC treatment by bevacizumab is accompanied by a 3-fold increase in FGF2 mRNA and protein level. We also observed an increase in FGFR1 mRNA and in FGFR1 phosphorylation (Fig. 3C). We then verified whether FGF2 was able to induce Akt activation and found that Akt phosphorylation could be detected in HUVEC as soon as 10 minutes after treatment with FGF2 (10 ng/mL; Fig. 3D, left). An overnight treatment with FGF2 shows that Akt phosphorylation in HUVECs is similar to the Akt phosphorylation observed in E4ECs as shown by phosphoflow analysis (Fig. 3D, right). Concordantly, the treatment of HUVECs by FGF2 induced resistance to bevacizumab and reversed the inhibition of angiogenic attributes (Supplementary Fig. S5A–S5C). We investigated the molecular events underlying the potential mechanisms of resistance through FGF2 expression in endothelial cells. As previously reported (28), endothelial FGF2 synthesis is regulated by a signaling cascade involving ERK1/2 and STAT3 activation. Under bevacizumab treatment, FGF2/FGFR1 axis mediates Src activation and downstream activation of the proangiogenic ERK1/2–STAT3 signaling pathway as shown by the increase phosphorylation of Src (Y416), STAT3, and ERK1/2 (Fig. 3E). The secretion of FGF2 has been mainly attributed to cancer cells (27, 29), we showed the existence of an autocrine loop...
maintaining Akt activation in E4⁺ ECs in the context of VEGF-A inhibition. Akt inhibition by LY294002 resulted in FGF-2 inhibition at mRNA and protein levels, suggesting that Akt activation is responsible for FGF2 secretion (Fig. 3F).

Inhibition of FGF2 signaling reversed bevacizumab resistance in E4⁺ ECs

To investigate whether the inhibition of FGF2 signaling would reverse bevacizumab resistance, we used a FGF2 blocking antibody. We showed that neutralization of FGF2 activity decreases Akt phosphorylation in E4⁺ ECs. We then showed a synergistic effect when anti-FGF2 was added to bevacizumab treatment. This result suggests that Akt activation of E4⁺ ECs depend on both VEGF-A and FGF2 secretion (Fig. 4A). We verified that blocking FGF2 alone did not impact E4⁺ EC survival. However, in the context of FGF2 deprivation, bevacizumab resistance was reverted (Fig. 4B). Finally, we also investigated the role of FGF2 in the angiogenic attributes. Blocking FGF2 alone has a minor effect on motility, but a drastic effect on tube formation, confirming the role of FGF2 as a potent angiogenic factor (Fig. 4C and D). We then assessed the effect of the inhibition of FGFR activity using 2 FGFR-selective inhibitors, PD17034 (30) and SU5402 (31) in combination with bevacizumab. While treatment with PD17034 (10 nmol/L) or SU5402 (1 μmol/L) alone resulted in a modest decrease of E4⁺ EC growth, a bevacizumab dose-dependent reduction of E4⁺ EC proliferation was observed after cotargeting VEGF-A and FGFR, confirming the synergistic role of the 2 cytokines (Fig. 4E). These data suggest that the FGF/FGFR axis influences the sensitivity of the E4⁺ ECs to bevacizumab therapy.

Residual disease

The rebound effect described in the literature and observed in the clinical setting can from an endothelial niche. We set up a model of coculture with endothelial and cancer cells and tried to reproduce a residual disease. Both HUVECs and E4⁺ ECs integrated tumor nodules of SKOV-3 produced on Matrigel. We then sequentially treated these nodules with the microtubule-damaging agent taxol (10 μmol/L) and bevacizumab (1 mg/mL). After taxol treatment, few cancer and endothelial cells survived. E4⁺ ECs presented significant resistance to the treatment. The treatment with bevacizumab completely eliminated all tumor and endothelial cells in the HUVEC control group. However E4⁺ ECs and SKOV-3 were able to grow and produce a “recurrent nodule” (Fig. 5A). We went further and created structures called angiospheres. We have recently illustrated the ability of the endothelium to form 3D structures that can sustain tissue-specific cell growth (32). We hypothesize that the same phenomenon could be relevant to cancer biology where within 3D structures the endothelial cells can support the expansion of the cancer-propagating cells. We were able to demonstrate an increase ability of cancer cells cocultured with E4⁺ ECs to form spheres comparing to cancer cells alone. The results were similar when secondary and tertiary spheres were induced (Fig. 5B). To confirm that E4⁺ ECs are responsible for the expansion of the spheroids under bevacizumab treatment, we treated daily the spheroids and showed that after 7 days, E4⁺ ECs can still support sphere formation (Fig. 5C).

Discussion

This study demonstrates that an endothelial FGF2 autocrine loop induces resistance to bevacizumab and leads to the creation of an Akt-dependent endothelial niche.

Angiogenesis supports tumor progression through delivery of oxygen and nutrients and provides a point of entry into circulation that enables blood-born metastases (33). Bevacizumab is the first antiangiogenic agent available in the clinical setting. Despite a number of clinical results and reports showing improvements in progression-free survival in patients with advanced ovarian cancer (34), some patients do not respond to bevacizumab or gradually develop resistance. Converging data support that tumor microenvironment modulates cancer cells sensitivity to treatment. Our study describes a comprehensive observational and functional investigation on the pivotal role played by the endothelium in the resistance to bevacizumab. Antiangiogenic agents are able to induce or aggravate hypoxia, which upregulates the production of other proangiogenic factors, leading to a "VEGF-independent" revascularization. This phenomenon is called evasive resistance (35). Le Page and colleagues reported that FGF2 levels are elevated in the serum of patients with ovarian cancer compared with cancer-free individuals and in tumoral tissue compared with nontumoral tissue (36). Concordantly, Lin and colleagues and Zhang and colleagues reported that FGF2 stimulates proliferation, migration, angiogenesis, and invasion in ovarian cancer cell lines OVCAR-3 and SKOV-3 (37–39). They also reported that treatment with an FGF2 antibody could inhibit FGF2-dependent proliferation and angiogenesis. Furthermore, Gan and colleagues reported that high FGF2 tumor levels reduced drug sensitivity, in part, due to the direct effects of FGF2 on proliferation and apoptosis (40). FGF2 activates the PI3K in endothelial cells (41), which regulate Akt or protein kinase B (PKB) activity (42). Our data indicate that FGF2 is able to revert the effect of bevacizumab on HUVECs and highlight FGF2 participation to E4⁺ EC survival under bevacizumab treatment. We demonstrated that FGF2 blocking would efficiently reverse the resistance to bevacizumab in E4⁺ ECs. We showed that primary OCCs and ovarian cancer cell lines express FGF2, these data support the FGF2/FGFR-mediated cross-talk between cancer and endothelium.

The cross-talk between the tumor and endothelium supports the tumor vasculature after bevacizumab
treatment in a paracrine manner. Butler and colleagues recently proposed that endothelial cells produce "angio-
crine" factors that could enable tumor growth, motility,
and ultimately metastasis (26). Autocrine circuit for FGF2
as well other angiocrine factors may be responsible for the
acquisition of autonomous proliferation of endothelial
cells in contact with the tumor. It has been suggested that
activated endothelium provides a vascular "niche"

Figure 5. Residual disease and regrowth of the nodule due to Akt-activated endothelium. A, residual disease model. Endothelial cells (E4+EC-eGFP and
HUVECs labeled with PKH26 tracker dye) were cocultured with SKOV-3 on top of growth factor–reduced Matrigel. The nodules were successively
treated with taxol (24 hours), followed by bevacizumab (5 days). Images of formed structures were taken by phase-contrast microscopy (4×; Taxol: 10
µmol/L, bevacizumab: 1.5 mg/mL). B, E4+EC-eGFP increase the ability of SKOV-3 to form 3D structures compared with SKOV-3 alone. Confocal
imaging representing cancer cell spheroids and the interaction of SKOV-3 with E4+EC-eGFP (angiospheres). Scale bar, 20 µm. Primary spheres (cancer
spheroids or angiospheres alone) were maintained up to 7 days. Pictures of the entire well were taken, and the surface area of SKOV-3 integrated in the
angiospheres or SKOV-3 spheroids alone was determined by ImageJ64 software. Secondary and tertiary spheres were produced as indicated in
Materials and Methods and the surface area measured as described. Graph bars display the values representing the mean ± the range of triplicate
determinations. C, E4+EC-eGFP supports sphere formation under bevacizumab treatment. E4+EC-eGFP and SKOV-3 dissociated into single-cell
suspension by trypsinization were suspended in 3D media in the presence or absence of bevacizumab (1.5 mg/mL). Spheroids were formed, and
bevacizumab was added daily to the culture during 7 days. E4+EC-eGFP survival was analyzed by ImageJ64 software based on the area covered by
E4+EC-eGFP spheroids. Graph bars display the values representing the mean ± the range of triplicate determinations (4×; scale bar, 500 µm).
* P < 0.05; ***, P < 0.001.
inducing the angiogenic switch in micrometastases and allowing the persistence of a residual disease. These data are compatible with our findings, as the activated endothelium resists to antiangiogenic therapy. The concept that a physical niche protects tumor cells during drug treatment has also been suggested for the perivascular space within the tumors. Indeed, recent work has highlighted a tumor reinitiating cells along tumor vessels, suggesting that these locations have a predictive role in tumor sensitivity to treatment (43, 44). Bissel group demonstrated that ECs and factors deposited within their surrounding basement membrane may be a prime player within the dormant niche and revealed that stable microvasculature constitutes a dormant niche, whereas sprouting neovasculature sparks micrometastatic outgrowth (45). Moreover, bevacizumab-treated spheroid cells could form more spheres than the control, indicating that they have an increased self-renew capacity. Patient’s therapeutic responses remain a significant challenge, and therapeutic resistance to bevacizumab reflects active tumor evolution and also microenvironmental-mediated resistance.

In summary, our study used the E4 ECs as a surrogate for tumor-associated endothelium. The tumor-protective effect of the endothelium can be attributed to the ability to support tumor vasculature after bevacizumab treatment, leading to the relapse of the tumor. An FGF2/PI3K/Akt autocrine loop is required in ECs to perturb bevacizumab treatment and involve the proangiogenic ERK1/2–STAT3 signaling pathway (Fig. 6). The FGF/FGFR/Akt signaling pathway is involved in an angiocrine switch and may be a target for therapeutic strategies against bevacizumab-resistant ovarian cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Disclaimer
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