ORIGINAL

Chronic exposure of VEGF inhibitors promotes the malignant phenotype of colorectal cancer cells

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Abstract : VEGF-targeting anti-angiogenic drugs have enabled significant advances in cancer therapy. However, acquired resistance to VEGF-targeting drugs occurs, leading to disease progression. How tumors become the resistance remains fully uncertain. One of possible mechanisms for the resistance may be the direct effect of VEGF inhibitors on tumor cells expressing VEGF receptors (VEGF-R). We investigated here the direct effect of chronic VEGF inhibition on phenotype changes in cancer cells. To chronically inhibit cancer cell-derived VEGF, human colon cancer HCT116 cells were chronically exposed (3 months) to anti-VEGF neutralizing monoclonal antibody (HCT/mAb cells, blockade of VEGF alone) or VEGF-R tyrosine kinase inhibitor foretinib (HCT/fore cells, blockade of all VEGF family). HCT/mAb cells redundantly increased VEGF family member (VEGF, PlGF, VEGF-B, VEGF-R1 and VEGF-R2) and induced a resistance to hypoxia-induced apoptosis. By contrast, HCT/fore cells did not show the redundant increase in VEGF family member, but significantly increased a VEGF-independent pro-angiogenic factor FGF-2. HCT/fore cells showed increased migration and invasion activities in addition to a resistance to hypoxia-induced apoptosis. The resistance to apoptosis was significantly suppressed by inhibition of hypoxia-inducible factor-1α in HCT/mAb cells, but not in HCT/fore cells. These findings suggest that chronic inhibition of VEGF/VEGF-R accelerates malignant phenotypes of colon cancer cells. J. Med. Invest. 62 : 75-79, February, 2015

Keywords **:** *VEGF, VEGF-targeting drugs, colon cancer cells, malignant phenotype*

INTRODUCTION

Vascular endothelial growth factors (VEGFs) and their tyrosine kinase receptors (VEGF-Rs) are central regulators of tumor angiogenesis and lymphangiogenesis. The VEGF family of growth factors include five members in mammals : VEGF (or VEGF-A), placenta growth factor (PlGF), VEGF-B, VEGF-C and VEGF-D. The VEGF ligands bind to three transmembrane tyrosine kinase receptors, VEGF-R1/Flt1, VEGF-R2/KDR and VEGF-R3/Flt4 (1-3).

VEGF is a crucial therapeutic target in cancer therapy because of its critical role in the induction of tumor angiogenesis (1-3). Several angiogenesis inhibitors targeting the VEGF-VEGF receptor pathway have been developed and become an important option for management of a number of human malignancies, including colorectal cancer. Unfortunately, a significant number of patients do not respond to VEGF-targeted therapy. Furthermore, the vast majority of patients who initially respond to anti-VEGF therapy will develop resistance (4-8). Therefore, inherently resistant and develop acquired resistance to the VEGF-pathway inhibitors is a growing concern in the clinic.

Mechanisms of the resistance include up-regulation of alternative pro-angiogenic factors (FGF-2, HGF and IL-8) and protection of the tumor vasculature either by recruiting proangiogenic proinflammatory cells or by increasing protective pericyte coverage (4-7). In addition to these proposed mechanisms, oncologists have begun to focus on the mechanisms of direct action of anti-VEGF agents on cancer cells, i.e., actions that are independent of the antiangiogenic effects of VEGF inhibitors, and tumor adaptation to VEGF inhibition (5).

In fact, VEGF-R is expressed not only in endothelial cells but also in several cancer cell lines, including colorectal, bladder, breast, and pancreatic cancer cells (9-13). In addition, an immunohistochemical screen of non-endothelial cancer specimens revealed detectable levels of VEGF-Rs in CRC, bladder, breast, and lung cancers (14). These observations suggested a possible autocrine/paracrine VEGF signaling pathway within cancer cells. Indeed, it has become clear that VEGF acts as an autocrine growth and survival factor for cancer cells that express VEGF-R (9-13). Thus, some of the effects observed with anti-VEGF therapies could result from "direct" effects on tumor cells.

However, the direct effects of anti-VEGF therapy on tumor cells are not yet fully understood. In this study, we examined the direct effects of VEGF-targeting agents on tumor cell phenotype. We also compared the effects on tumor cells between anti-VEGF neutralizing monoclonal antibody (anti-VEGF mAb, blockade of VEGF alone) and VEGF-R tyrosine kinase inhibitor foretinib (blockade of all VEGF family ligands ; VEGF, VEGF-B, PlGF and VEGF-C). We found that chronic exposure of colon cancer HCT116 cells to anti-VEGF mAb resulted in a resistance to hypoxia-induced apoptosis. Intriguingly, chronic exposure to foretinib induced not only a marked resistance to hypoxia-induced apoptosis, but also enhancement of migration activity. These results provide a new insight into the adaptation of colon cancer cells to the loss of VEGF signaling.

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MATERIALS AND METHODS

Cell culture and treatment

Human colon cancer cell line (HCT116) was maintained in RPMI1640 medium with 10% fetal bovine serum and antibiotics. To develop the HCT/mAb and HCT/fore cell lines, HCT116 cells were chronically exposed to anti-VEGF mAb (10μg/ml, R&D Biosystems) or VEGF-R tyrosine kinase inhibitor (10 nM foretinib, Selleckchem) for 60 days in RPMI1640 medium with 10% fetal bovine serum and antibiotics.

Hypoxic treatment and HIF-1α-dependent transcriptional activity

For hypoxic culture conditions, cells were incubated at low confluence and 37°C in BBL GasPak 100 anaerobic system in which O_2 was ~0.2% (BD Biosciences). Hypoxic treatment was functionally confirmed by transactivation of HIF-1α using a HIF-1α-dependent reporter construct combined with internal control reporter construct (Cignal HIF reporter assay kit, SA Biosciences).

Quantitative RT-PCR (qRT-PCR)

The levels of transcripts for VEGF ligands (*Vegf-a*, *Vegf-b*, and *Plgf*), VEGF receptors (*Vegfr1* and *Vegfr2*), β-actin were measured by real time (RT)-PCR using the following specific primer sets : *Vegf-a*, 5′-GAGCCTTGCCTTGCTGCTCTAC-3′ (forward) and 5′-CACCAGGGTCTCGATTGGATG-3′ (reverse) ; *Vegf-b*, 5′-CTGGCCACCAGAGGAAAGT-3′ (forward) and 5′-CATGAGCTCCACAGTCAAGG-3′ (reverse) ; *Plgf*, 5′-GGCTGTTCCCTTGCTTCC-3′ (forward) and 5′-CAGACAAGGCCCACTGCT-3′ (reverse) ; *Vegfr1*, 5′-AGAACCCCGATTATGTGAGAAA-3′ (forward) and 5′-GATAGATTCGGGAGCCATCC-3′ (reverse) ; *Vegfr2*, 5′-GAACATTTGGGAAATCTCTTGC-3′ (forward) and 5′-CGGAAGAACAATGTAGTCTTTGC-3′ (reverse) ; β-actin, 5′-CCAACCGCGAGAAGATGA-3′ (forward) and 5′-CCAGAGGCGTACAGGGATAG-3′ (reverse). Amplification and quantification of the PCR products were performed using the Applied Biosystems 7500 System (Applied Biosystems). Standards were run in the same plate and the relative standard curve method was used to calculate the relative mRNA expression. RNA amounts were normalized against the β-actin mRNA level.

Assessment of apoptosis

Apoptotic cells were assessed by a DeadEnd TUNEL-staining kit (Promega), as previously described (23).

Cell migration and invasion assay

Migration assay was conducted as described by Fan *et al* (9) with minor modifications. Equal numbers (50,000 cells per well) of cells were suspended in 0.25 ml of 1% RPMI1640-FBS without or with anti-VEGF mAb or foretinib and placed in the top compartment of a 8 μm pore membrane chambers (BD Biosciences) ; 0.75 ml of 10% RPMI1640-FBS was added to the bottom compartment. Following 24-h incubation under standard conditions (37°C/5% CO_2), non-migrating cells were scraped from the top compartment, and cells that had migrated to the bottom compartment were fixed and stained using the Hemacolor Rapid staining of blood smear (Merck). Membranes were excised and mounted on a standard microscope slide. The numbers of migrated cells were determined from five random high-power fields visualized at×200 magnification.

Invasion assays were done using a similar protocol with minor modifications. The inserts used in the invasion assays were coated with Matrigel (BD Biosciences) and prehydrated with 1% FBS-supplemented medium for 2 hours before the addition of the cell suspension. Following 48-h incubation under standard conditions (37°C/5% CO_2), and the numbers of invading cells were again quantified.

Statistical analysis

Results are expressed as means± S.D. Statistical analyses of data were done using ANOVA and the Scheffé's test. P values< 0.05 was considered significant.

RESULTS

Effect of chronic treatment with anti-VEGF mAb or foretinib on the expression of VEGF family members

It has been demonstrated that blockade of VEGF by anti-VEGF mAb redundantly increase in expression of VEGF family members (12). Thus, we first examined whether chronic loss of VEGF signalling (VEGF alone by anti-VEGF mAb or all of VEGF ligands by foretinib) induces the redundant expression of VEGF family members. To adapt to the VEGF inhibitors, HCT116 cells were chronically treated for 60 days with anti-VEGF mAb (HCT/mAb), with foretinib (HCT/fore), or without any treatment (HCT/par). The expression levels of VEGF ligands (VEGF, PlGF and VEGF-B) and VEGF-Rs (VEGF-R1 and -R2) were measured by RT-qPCR. HCT/mAb cells increased the expression of all of VEGF ligands and VEGF-Rs tested (approximately 2- to 2.5-fold) relative to the control HCT/par cells (Figure 1A-E). In contrast, HCT/fore cells did not increase all VEGF ligands and receptors tested (Fig. 1A-E). Intriguingly, HCT/fore cells, but not HCT/mAb cells, increased

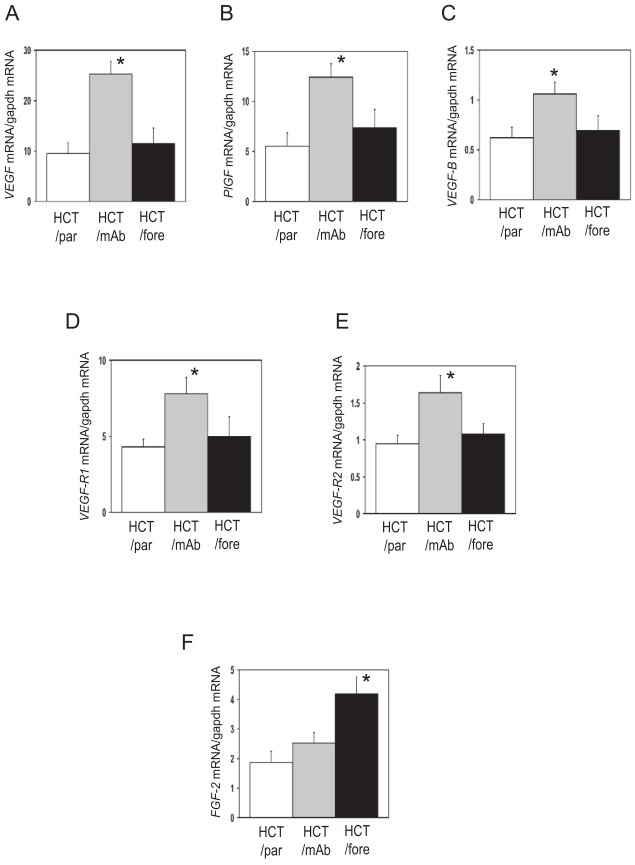

Figure 1. Effect of chronic exposure with anti-VEGF mAb or foretinib on VEGF family and FGF-2 expression.
HCT116 cells were chronically treated with vehicle (HCT/par) or with anti-VEGF mAb (HCT/mAb) or foretinib (HCT/fore) for 90 days. Expression levels of VEGF (A), *PlGF* (B), *VEGF-B* (C), *VEGF-R1* (D), *VEGF-R2* (E), and *FGF-2* (F) were measured by quantitative RT-PCR (n=4-5, means± S.D.). **P*< 0.01., compared with control HCT/par cells.

FGF-2 expression (Fig. 1F) that is well characterized as pro-angiogenic factor under VEGF-inhibited conditions (5). These results suggest that prolonged blocking of all VEGF family switched VEGF-dependent phenotype to VEGF-independent one.

Effect of chronic treatment with anti-VEGF mAb or foretinib on hypoxia-induced apoptosis

As one of the major *in vivo* effects of VEGF inhibition is anti-angiogenesis and its contribution to tumor hypoxia, we examined sensitivity to hypoxia-induced apoptosis in HCT/mAb and HCT/fore cells. After exposure to hypoxic conditions (~ 0.2% O_2) for 48 h, control HCT/par cells displayed a heightened degree of apoptosis (Fig. 2A). HCT/mAb showed a resistance to hypoxia-induced apoptosis (Fig. 2A). HCT/fore cells exhibited a marked resistance to the apoptosis, compared with HCT/mAb cells.

To explore how HCT/mAb and HCT/fore cells became resistant to hypoxia-induced apoptosis, we focused on HIF-1α, since HIF-1α is a critical regulator of many hypoxia responses, including resistance to apoptosis (15, 16). We confirmed that HIF-1α transcriptional activity under hypoxic conditions was significantly increased in HCT/mAb and HCT/fore cells compared with control HCT/par cells (Fig. 2B). We used a HIF-1α inhibitor (FM19G11) that effectively inhibited HIF-1α transcriptional activity under hypoxic conditions in HCT/par, HCT/mAb and HCT/fore cells (Fig. 2B, gray bar). Inhibition of HIF-1α activity in HCT/mAb cells significantly increased hypoxia-induced apoptosis. However, blocking of HIF-1α activity in HCT/fore cells did not increase hypoxia-induced apoptosis (Fig. 2C, gray bar).

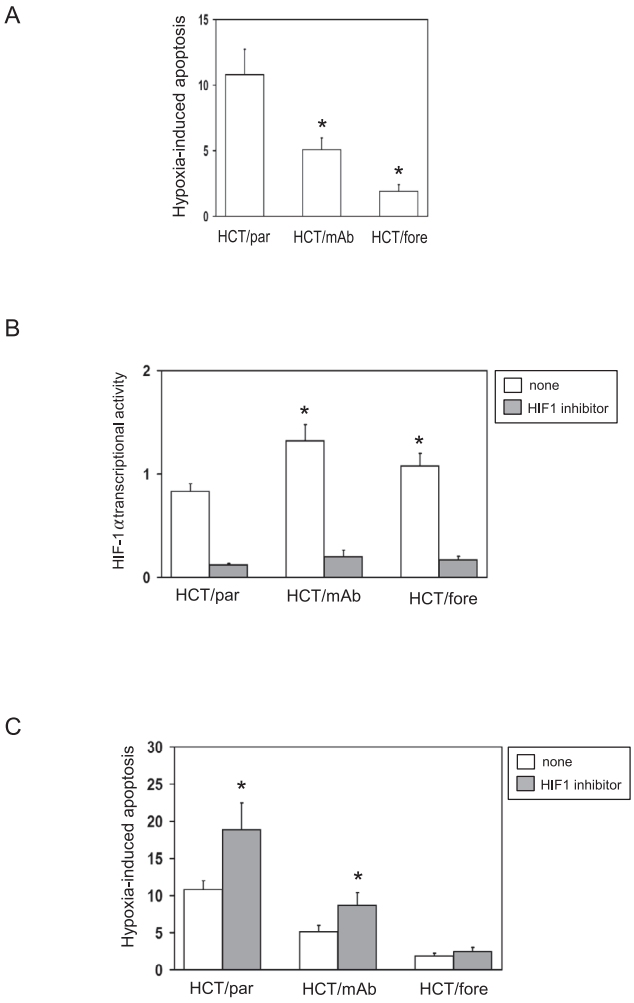

Figure 2. Effect of chronic exposure with anti-VEGF mAb or foretinib on hypoxia-induced apoptosis.
(A) Cells were exposed to hypoxia (~ 0.2% O_2) for 48 h, then apoptotic cells were determined by TUNEL assay (n=4-5, means± SD). *P< 0.01., compared with control HCT/par cells.
(B) Transcriptional activity of HIF-1α under hypoxic conditions without (none) or with HIF-1 inhibitor. Cells were transfected with a HIF-1α-dependent reporter construct (LucF) and a internal control reporter plasmid (LucR), then they were exposed to hypoxia (~ 0.2% O_2) for 24 h. The transcriptional activity of HIF-1α was determined by a dual luciferase assay (n=4, means± SD). *P< 0.01., compared with control HCT/par cells.
(C) Cells were exposed to hypoxia (~ 0.2% O_2) for 48 h in the absence (none) or presence of HIF-1 inhibitor, then apoptotic cells were determined by TUNEL assay (n=6, means± SD). *P< 0.01., compared with the respective control cells (none) in each group.

Effect of chronic treatment with anti-VEGF mAb or foretinib on cell migration and invasion

We then assessed the effects of anti-VEGF mAb or foretinib on cell migration activity. HCT/mAb cells showed a slight increase in migration compared with the control HCT/par cells (Fig. 3A, B). By contrast, HCT/fore cells showed 10-fold increase in migration activity (Fig. 3A, B).

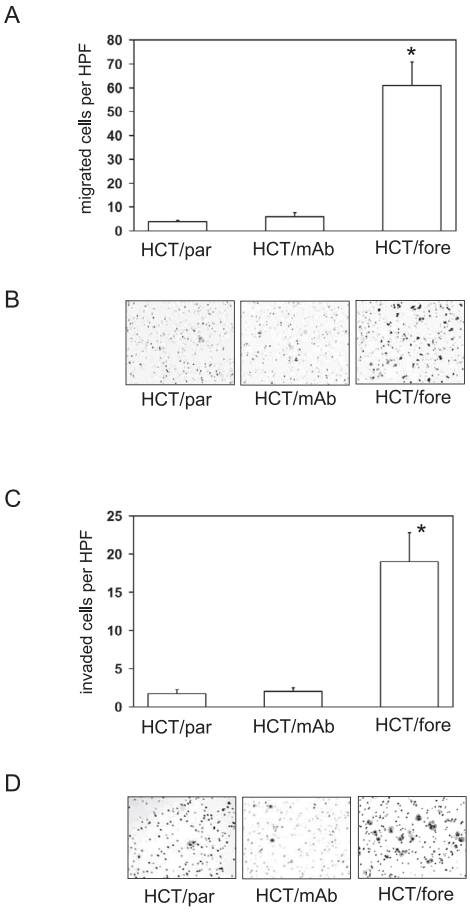

Figure 3. Effect of chronic exposure with anti-VEGF mAb or foretinib on migration and invasion activity.
(A) HCT/par, HCT/mAb and HCT/fore cells were used for transwell migration assay (n=4, means± SD). HPF, high power field. *P< 0.01., compared with control HCT/par cells.
(B) Photographs of migrated cells.
(C) HCT/par, HCT/mAb and HCT/fore cells were used for transwell invasion assay (n=4, means± SD). *P< 0.01., compared with control HCT/par cells.
(D) Photographs of invaded cells.

We also examined the effects of anti-VEGF mAb or foretinib on cell invasion activity. In agreement with migration activity, HCT/fore cells showed an increased invasion activity (Fig. 3C, D). There was no difference in a low invasion activity between HCT/mAb and HCT/par cells (Fig. 3C, D).

DISCUSSION

This study focused on the direct and chronic effects of VEGF inhibition on tumor cells using two cell models (HCT/mAb cells and HCT/fore cells). We found that chronic inhibition of VEGF alone resulted in resistance to hypoxia-induced apoptosis, while chronic blockade of all VEGF ligands induced more aggressive phenotypes (not only apoptotic resistance but also migration/invasion phenotype).

In response to chronic blockade of VEGF, redundant expression of VEGF family members (VEGF, PlGF, VEGF-B, VEGF-R1 and -R2) was observed in HCT/mAb cells. Many studies have similarly shown that inhibition of VEGF signaling *in vitro* or *in vivo* leads to compensatory increases in the expression of VEGF family ligands (5, 12). By contrast, chronic blockade of all VEGF ligands signaling by foretinib did not show the redundancy, suggesting that HCT/fore cells acquire VEGF-independent phenotype. In fact, HCT/fore cells, but not HCT/mAb cells, increased expression of other pro-angiogenic factor FGF-2.

Both HCT/mAb and HCT/fore cells acquired an apoptosis resistance induced by hypoxic stress. One of possible adaptive mechanisms may involve a HIF-1α activation. Many studies have established critical roles for HIF-1α in tumor cell survival and malignancy : i) HIF-1α is involved in repression of hypoxia-induced apoptosis *in vitro* (16) ; ii) HIF-1α plays important roles in resistance to VEGF inhibitor (17-19). In agreement with these reports, HCT/mAb cells required HIF-1α activation for their resistance to apoptosis. However, HCT/fore cells did not require HIF-1α, suggesting that HCT/fore cells activate other adaptive pathway that has yet remained undefined.

Based on the present data and recent reports, it is possible that anti-VEGF therapies directly inhibit VEGF signaling in tumor cells, which may remodel tumor cell survival signal(s). In fact, recent report clearly showed that VEGF suppresses migration of tumor cells *in vitro* (21) ; inhibition of VEGF signaling conversely accelerated migration and invasion *in vivo* (21). These findings suggest that over the long term inhibition of VEGF, such remodeling results in adaptation to VEGF inhibition, and this adaptive response may represent one of potential mechanism of acquired resistance to anti-VEGF therapies.

VEGF initially held great promise as a therapeutic target. In fact, VEGF-targeting therapy has been shown to be very effective in certain tumor types, such as renal cell carcinoma (22, 23). However, the overall benefit of blocking VEGF activity in other solid tumors is marginal and has led to some skepticism in the field. Therefore, molecular mechanism(s) activated by chronic loss of VEGF signaling will need to be elucidated to improve and further develop VEGF-targeting therapies.

COMPETING INTERESTS-DISCLOSURE

The authors declare no competing interests.

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