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VEGFR2 Signaling Prevents Colorectal Cancer Cell Senescence to Promote Tumorigenesis in Mice With Colitis

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BACKGROUND & AIMS: Senescence prevents cellular transformation. We investigated whether vascular endothelial growth factor (VEGF) signaling via its receptor, VEGFR2, regulates senescence and proliferation of tumor cells in mice with colitis-associated cancer (CAC). **METHODS:** CAC was induced in VEGFR2$^{\Delta IEC}$ mice, which do not express VEGFR2 in the intestinal epithelium, and VEGFR2$^{fl/fl}$ mice (controls) by administration of azoxymethane followed by dextran sodium sulfate. Tumor development and inflammation were determined by endoscopy. Colorectal tissues were collected for immunoblot, immunohistochemical, and quantitative polymerase chain reaction analyses. Findings from mouse tissues were confirmed in human HCT116 colorectal cancer cells. We analyzed colorectal tumor samples from patients before and after treatment with bevacizumab. **RESULTS:** After colitis induction, VEGFR2$^{\Delta IEC}$ mice developed significantly fewer tumors than control mice. A greater number of intestinal tumor cells from VEGFR2$^{\Delta IEC}$ mice were in senescence than tumor cells from control mice. We found VEGFR2 to activate phosphatidylinositol-4,5-bisphosphate-3-kinase and AKT, resulting in inactivation of p21 in HCT116 cells. Inhibitors of VEGFR2 and AKT induced senescence in HCT116 cells. Tumor cell senescence promoted an anti-tumor immune response by CD8$^+$ T cells in mice. Patients whose tumor samples showed an increase in the proportion of senescent cells after treatment with bevacizumab had longer progression-free survival than patients in which the proportion of senescent tumor cells did not change before and after treatment. **CONCLUSIONS:** Inhibition of VEGFR2 signaling leads to senescence of human and mouse colorectal cancer cells. VEGFR2 interacts with phosphatidylinositol-4,5-bisphosphate-3-kinase and AKT to inactivate p21. Colorectal tumor senescence and p21 level correlate with patient survival during treatment with bevacizumab.

Keywords: Angiogenesis; Colon Cancer; Mouse Model; Inflammation.

Carcinogenesis of the colorectum is a multistep process that involves initiation, promotion, and, finally, progression into invasive carcinoma.[1,2] Recently, the vascular endothelial growth factor (VEGF) pathway moved to the center of attention during inflammation-associated colorectal carcinogenesis. Generally, VEGF is regarded as the key mediator of tumor angiogenesis.[3] The main effector is the VEGF receptor 2 (VEGFR2), which is expressed by endothelial cells (ECs). Activation of VEGFR2 on ECs results in their proliferation, migration, and increased survival.[4] Consequently, strategies blocking VEGF signaling were able to reduce tumor growth in multiple studies, which led to the approval of bevacizumab (anti-VEGF antibody) for the treatment of human cancer.[5]

Growing evidence supports the role of VEGF as an autocrine, paracrine, and even "intracrine" growth factor for tumor cells themselves, independent from its role in angiogenesis. Among others, this was studied for breast cancer cells,[6] skin cancer cells,[7] CD133$^+$ glioblastoma stem cells,[8] and in colitis-associated carcinogenesis.[9] Regarding colitis-associated cancer (CAC), we could show that VEGFR2 is up-regulated on intestinal epithelial cells (IECs) during acute and chronic inflammation, and its activation increases tumor promotion and proliferation. Although these results prompt VEGFR signaling to be an important molecular link between inflammation and colorectal cancer (CRC), details on the underlying mechanisms are scarce.

Using conditional knockout mice for VEGFR2 (VEGFR2$^{\Delta IEC}$) for the first time, we show that VEGF/VEGFR2 signaling plays a crucial role in inflammation-associated carcinogenesis by bypassing cellular senescence in IECs and promoting tumor development and progression. VEGFR2$^{\Delta IEC}$ mice were significantly protected against inflammatory carcinogenesis, and VEGFR2$^{\Delta IEC}$ tumors displayed a senescent phenotype. On the molecular

Abbreviations used in this paper: **AOM, azoxymethane; ATIR, anti-tumor immune response; CAC, colitis-associated cancer; CRC, colorectal cancer; DSS, dextran sodium sulfate; EC, endothelial cells; IEC, intestinal epithelial cell; IHC, immunohistochemistry; MVD, microvessel density; PI3K, phosphatidylinositol-4,5-bisphosphate-3-kinase; qPCR, quantitative polymerase chain reaction; Sen-β-Gal, senescence-associated β-galactosidase; VEGF, vascular endothelial growth factor; VEGFR2, vascular endothelial growth factor receptor 2.**

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level, autocrine VEGF/VEGFR2 signaling in CRC cells seems to lead to an activation of the phosphatidylinositol-4,5-bisphosphate-3-kinase (PI3K)/AKT cascade, with subsequent inactivation and degradation of $p21^{WAF1/Cip1}$, a central molecule in senescence induction and maintenance (further referred to as p21). In addition, systemic VEGFR2 inhibition reduced tumor growth and enabled cellular senescence in experimental CAC. This effect was functionally dependent on both the induction of senescence and subsequent adaptive immune response. In addition, the ability to induce tumor cell senescence was associated with improved progression-free survival in bevacizumab-treated CRC patients.

Our findings highlight a central role of VEGF signaling independent from angiogenesis, and suggest an unknown connection between escape from aging-associated safeguard programs and inflammation-associated carcinogenesis.

Materials and Methods

Animal Models

Animal experiments were approved by the Institutional Animal Care and Use Committee of the State Government of Middle Franconia and conformed to national and international guidelines. $VEGFR2^{fl/fl}$ mice, Villin-Cre mice, and $p21^{-/-}$ mice have been described and were bred on a C57BL/6 background.[10–13] $VEGFR2^{fl/fl}$ mice were crossbred with Villin-Cre mice to generate $VEGFR2^{\Delta IEC}$ mice lacking the VEGFR2 in their IECs. All animals used were 8- to 10-week-old females (if not indicated otherwise). Azoxymethane (AOM)+dextran sodium sulfate (DSS) colorectal carcinogenesis was induced as described previously.[14]

Endoscopy, Narrow-Band Imaging, and Full-Body Fluorescence Imaging

High-definition endoscopy was achieved using specialized endoscopes, which have been described recently by our group.[15] Severity of colitis, tumor count, and tumor mass were determined using an established endoscopic scoring system.[16] Narrow-band imaging was used for vessel characterization.

Senescence-Associated β-Galactosidase Assay

Senescence-associated β-galactosidase (Sen-β-Gal) staining was performed using the Cell Signaling staining kit with a modified protocol (Cell Signaling Technology, Danvers, MA). Briefly, for tissue staining, colonic and tumorous tissues were removed, snap frozen in liquid nitrogen, and cryosectioned within 15 minutes after removal. Sen-β-Gal solution adjusted for pH 6 and the tissue was incubated overnight at 37°C.

Administration of Vascular Endothelial Growth Factor Receptor 2 Inhibitor, $CD8^+$ Depletion Antibody

Treatment of C57BL/6 and $p21^{-/-}$ mice with VEGFR2 inhibitors, $CD8^+$ T-cell depletion was initiated after endoscopic detection of already established tumors at week 4 of the AOM+DSS protocol.

Results

Loss of Vascular Endothelial Growth Factor Receptor 2 Signaling in Intestinal Epithelial Cells Protects Against Tumor Development in an Experimental Model of Colitis-Associated Cancer

To investigate the functional role and the underlying molecular mechanisms of VEGFR2 signaling in CAC, we generated a conditional knockout for this receptor in IECs by crossbreeding mice with floxed VEGFR2 alleles and mice expressing the Cre-recombinase under the control of the intestinal villin promoter (referred to as $VEGFR2^{\Delta IEC}$; Figure 1*A*). Specific deletion of VEGFR2 was confirmed by quantitative polymerase chain reaction (qPCR) of IEC complementary DNA after inflammatory challenge with DSS (Supplementary Figure 1*A*). Immunofluorescence staining revealed a lack of receptor expression in IECs when compared with $VEGFR2^{fl/fl}$ mice (control, Figure 1*B*). Endoscopy of $VEGF2^{\Delta IEC}$ mice revealed no spontaneous phenotype in the lower gastrointestinal tract, which was confirmed by histology (Supplementary Figure 1*B*).

To induce colitis-associated tumor development, $VEGFR2^{\Delta IEC}$ and matched $VEGFR2^{fl/fl}$ control mice were exposed to AOM+DSS (Figure 1*C*).[17,18] Colonoscopy was performed to evaluate different tumor parameters and the severity of intestinal inflammation.[16] $VEGFR2^{\Delta IEC}$ mice showed significantly fewer tumors and a significantly lower tumor load, as compared with control mice. Mean tumor size also differed, but did not reach statistical significance (Figure 1*D*).

Previous studies had suggested that the absence of VEGF reduces the severity of intestinal inflammation in an acute model of colitis and therefore might influence tumor development.[19] However, there was no significant difference in endoscopic activity of chronic DSS colitis between $VEGFR2^{\Delta IEC}$ and control mice (Figure 1*E*). We further investigated levels of oxidative stress as a possible initiator of tumor development. Immunohistochemistry (IHC) for the surrogate marker 8-hydroxydesoxyguanosine showed an increase in inflamed colonic tissue when compared with healthy control tissue from control mice (Figure 2*A*). This was further demonstrated by full-body fluorescence imaging using a fluorescent probe for reactive oxygen species detection (ROS Brite 700 nm; AAT Bioquest, Sunnyvale, CA) (Figure 2*B*). However, there was no difference in mucosal 8-hydroxydesoxyguanosine expression between $VEGFR2^{\Delta IEC}$ and control mice.

As these findings excluded the possibility that the differences in tumorigenesis between $VEGFR2^{\Delta IEC}$ and control mice were due to changes in colitis activity, we assessed the role of angiogenesis.[4] We performed CD31 staining of tumor tissue of both groups and calculated microvessel density (MVD). No differences in MVD could be observed. Likewise, narrow-band imaging endoscopy and full-body fluorescence imaging studies using a vessel-specific fluorescent probe ($\alpha V\beta 3$-integrin) showed no difference in overall macroscopic vascularity (Figure 2*C* and *D*). We concluded that differences in tumor development, growth, and morphology