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Transgenic Expression of VEGF in Intestinal Epithelium Drives Mesenchymal Cell Interactions and Epithelial Neoplasia

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Abstract

Background & Aims—Vascular endothelial growth factor (VEGF) is expressed robustly in human colon neoplasia and is a major new ‘rational’ target of therapy for cancers of the colon and other organs. Nonetheless, the mechanism(s) of action of VEGF-targeted therapies and the biological roles of VEGF in tumorigenesis have not been well defined. We used a transgenic approach to directly test the hypothesis that augmented VEGF expression can drive progression of intestinal neoplasia.

Methods—Transgenic mouse lines were generated with moderate (vilVEGF1) and high (vilVEGF2) VEGF expression from the intestinal epithelium. vilVEGF1 mice were bred to Min mice (Adenomatous polyposis coli (APC) +/-). Colon epithelial cells from an APC patient were co-cultured with endothelial cells and fibroblasts.

Results—vilVEGF mice were generally healthy but displayed red small intestines. Vessels were larger and more numerous in the submucosa but not the mucosa. The mucosa showed striking stromal and epithelial hypercellularity, with increased epithelial proliferation. Many crypts formed cysts composed of relatively undifferentiated epithelial cells surrounded by cells with endothelial and myofibroblast markers. Compared to Min controls, vilVEGF1-Min mice developed 6-fold more intestinal adenomas of all sizes, with more advanced histological features. Polycystic masses were also observed. Co-culture of human colonocytes with endothelial cells and fibroblasts directly stimulated colonocyte proliferation.

Conclusions—Augmented VEGF expression from intestinal epithelium potently stimulated crosstalk with mesenchymal cells and proliferation of normal and neoplastic epithelium. These effects of VEGF, largely occurring prior to the canonical angiogenic switch in tumors, may be in part independent of angiogenesis.

Vascular endothelial growth factor (VEGF(A)) has emerged as a major target of ‘rational’ cancer therapy (1). The goal of rational therapy is to selectively antagonize key factors

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deregulated in cancer. In contrast, traditional cytotoxic chemotherapy, the mainstay of cancer treatment, relies on agents that broadly kill proliferating cells. Expression of VEGF is increased in many solid tumors, including colon tumors (2,3). Antibodies directed against VEGF have proven to be useful when administered in combination with cytotoxic agents in treatment of advanced colon, breast, and non-small cell lung cancers (1,4-6). Nonetheless, these antibodies have been relatively ineffective as single agents, and their mechanism of action has remained unclear (7).

Most prominent among its properties, VEGF fosters angiogenesis, a process thought necessary for growth of many solid tumors beyond the limit of diffusion of oxygen (about 1-3 mm) (8, 9). The success of anti-VEGF therapy has been widely interpreted as a success for anti-angiogenic therapy. Known also as vascular permeability factor, VEGF augments extravasation of fluid and cells from vessels, a property that can increase hydrostatic pressure in tumors, potentially hampering tumor penetration by circulating drugs. Thus, an alternative mechanism by which anti-VEGF antibodies might achieve therapeutic benefit in combination chemotherapy is to reduce the hydrostatic pressure in tumors, improving drug penetration (7). This theory can account for the limited utility of anti-VEGF antibodies as single agents. VEGF receptors are present on vascular endothelial cells, where they contribute to endothelial cell survival, proliferation, mechanical properties, and migration (10). Recent evidence has been obtained for autocrine stimulation of proliferation of some tumor cells by VEGF, through receptors that appear to be induced in malignancy (7). This observation provides another potential role for VEGF in tumor growth. Clarification of the roles of VEGF in tumorigenesis is needed to further rationalize use of VEGF-targeted agents and design new therapies.

We have studied the mechanisms of suppression of colon tumorigenesis by p16^{Ink4a}. p16-null Multiple intestinal neoplasia (Min) mice show accelerated tumor progression, associated with increased vascularity and VEGF levels (11,12). Acute p16 expression represses VEGF in human colon cancer cells in vitro (11), and conditional deletion of the VEGF gene results in reduced mammary epithelial cell proliferation and p16 induction (13), raising the possibility of bidirectional regulatory loops.

To investigate the role of VEGF in intestinal tumorigenesis, we engineered transgenic mice in which VEGF expression is enhanced in the intestinal epithelium. We find that VEGF broadly stimulates formation of non-neoplastic tissue as well as pre-tumorous and tumorous neoplasia.

Materials and Methods (see Supplementary Material for more detail)

Transgenic mice

The cDNA for murine VEGF₁₆₅ was amplified by PCR from the pEF2-VEGF plasmid (kindly provided by W. Lee, University of Pennsylvania) using primers encoding 5-prime XhoI and 3-prime KpnI restriction sites and cloned into the polylinker of the p12.4 kb villin plasmid (kindly provided by D. Gumucio, University of Michigan). Transgenic mice were generated by standard pronuclear DNA injection of the purified eukaryotic sequences.

Tissue staining, Cell culture, VEGF assays

Standard procedures were used for tissue staining, as previously described (14). Pockets of RBCs were defined by the presence of a cluster of 3 or more nearby RBCs. Mouse intestines do not show a distinct muscularis mucosae. Therefore, we defined the submucosa as below the bottom of crypts and above the muscularis propria. In measuring the length of the columns of BrdU-positive epithelial cells, columns were avoided whose shape was distorted by neighboring cysts in vilVEGF mice. Organotypic cultures were generated by embedding cells in a collagen matrix, as previously described, with modifications (15). VEGF was assayed by

enzyme-linked immunosorbent (ELISA) assay (R&D Systems, Minneapolis, MN) and real-time reverse-transcription (RT)-PCR.

Statistical Methods

Ki67 labeling was analyzed using Generalized Estimating Equations and T-test of the means, with similar results. Other histological features were compared by Wilcoxon 2-sample tests. The distribution of tumor sizes in range bins and advanced features in tumors were compared by Fisher's exact test. To compare organotypic cultures, the Wilcoxon 2-sample test was used and an overall P-value was established by chi-squared analysis.

Results

Generation of vilVEGF transgenic mice

We cloned the cDNA for the major 165 amino acid splice variant of mouse VEGF (16) under the direction of the villin promoter (D. Gumucio, University of Michigan, Ann Arbor, MI) (17). This promoter has been shown to mediate transgene expression throughout the small and large intestines, with minimal activity in other tissues (some in the kidney) (17). Expression occurs in all intestinal epithelial cell types, from crypts to the luminal surface. VEGF₁₆₅ is among the most broadly expressed forms, evinces the most potent angiogenic activity in tumors, and is increased in human colon adenomas and cancer (3,16,18). After confirming the sequence of our 'vilVEGF' construct, we demonstrated by ELISA that it could direct secretion of VEGF into the culture medium of transfected HCT116 cells (data not shown). The secreted VEGF bound soluble VEGF receptor (Flt-1) produced by co-transfection (data not shown).

From about 100 potential founders, four were found to carry the transgene, a somewhat low frequency. Two died before producing offspring and could not be analyzed. In initial studies of the first transgenic line established ('vilVEGF1'), we noted increased embryonic and adult mortality during inbreeding into a C57Bl/6 background. 7 of 28 transgenic adults developed rectal prolapse. Others had bloody stools. For subsequent experiments, we crossed vilVEGF1 mice to C57Bl/6/Balb hybrid mice with fecundity and survival approaching normal. The intestinal histopathology (see below) was similar in both backgrounds. vilVEGF2 mice were maintained from the outset in the mixed genetic background, with only modestly reduced survival (data not shown).

Upon dissection, wild type (WT) small intestines were tan colored. In contrast, small intestines from vilVEGF1 mice were subtly pink (data not shown) and those of vilVEGF2 mice were consistently red (Figure 1). Homozygous vilVEGF1 small intestines were also consistently red, though less so than vilVEGF2 heterozygotes. These phenotypes suggested increased vascularity due to VEGF expression, with higher levels in vilVEGF2 than vilVEGF1 mice. Real-time RT-PCR assay of VEGF mRNA indeed demonstrated moderately higher levels in vilVEGF1 and markedly higher levels in vilVEGF2 intestine than non-transgenic tissue (Figure 2A). VEGF ELISA assays of intestinal mucosal scrapings demonstrated similar increases in VEGF protein (Figure 2B). Expression in vilVEGF2 mice was highest in the small intestine, consistent with the typical pattern of villin promoter activity (17) and the increased red color of this intestinal region. The relative increase in VEGF expression in transgenic mice was greatest in the colon, where endogenous VEGF levels were lowest. We achieved large numbers of vilVEGF1 mice sooner. In addition, the 2-8 fold higher mucosal VEGF levels in this line are comparable to the differences observed by ELISA between p16-null and p16-wt Min intestinal tumors and between Min tumors and non-tumor tissue, respectively (11,12,19). We therefore focused most subsequent studies on this line.

Intestinal mucosal thickening and cyst formation

A survey of non-intestinal organs from vilVEGF mice, including brain, lung, heart, muscle, kidney, liver, spleen, and stomach, revealed no gross anatomic or histological pathology (data not shown). Histological analysis of intestine sections from vilVEGF1 and vilVEGF2 mice revealed marked mucosal thickening punctuated by unusual cysts (Figure 3). These cysts were seen in every vilVEGF mouse examined (>40) and not in normal mice, suggesting that they are specific to the transgene and that transgenic VEGF expression was achieved in every mouse. In some cases cysts were seen to originate from crypts, in contiguity with the epithelial sheets (Figure 3, center). Immunohistochemical (IHC) staining for the epithelial marker Claudin-7 (Figure 4A,B), and E-cadherin (data not shown) confirmed that the cysts contained an inner lining of epithelial cells. Some cysts were found to be contiguous with multiple crypts (data not shown) or appeared to be dividing (Figure 4B), consistent with evidence for crypt division in normal mucosa (20). Staining with antibodies directed against CD34 (Figure 4C-E) and CD31 (data not shown), both markers of bone-marrow-derived cells and endothelial cells, suggested that the epithelium of most cysts was surrounded by an endothelial cell layer. Some of these cells also stained for von Willebrand Factor (vWF; data not shown; supplementary Figure 1). Such cells are enriched in the region below the crypts in WT mice (Figure 4C). Staining for α -smooth muscle actin (α -SMA), a myofibroblast marker, was also largely confined in WT tissues to the stroma beneath crypts and around mature blood vessels but surrounded many mucosal cysts in vilVEGF1 mice (Figure 4F, data not shown). Co-immunofluorescent staining indicated that the α -SMA-positive cells were distinct from the CD34-labeled cells (Figure 4F). Thus, these studies demonstrated robust VEGF-mediated recruitment to the intestinal epithelium of mesenchymal cells normally found around vessels and the base of intestinal crypts.

Cells of the cyst epithelial layer farther from the crypt base demonstrated less differentiation (lack of polarity, claudin-7, and E-cadherin staining; Figure 4A,B, data not shown). An occasional goblet cell was found in the cyst epithelial layer (Figure 3, center). Cyst interiors were composed of mucous (Alcian blue staining not shown) and dying or dead cells (activated caspase 3 staining not shown). At the far wall of cysts, lining cells were often observed peeling off into the interior (Fig 3, data not shown).

Hematoxylin and eosin, CD34, CD31, α -SMA, and vWF stains in vilVEGF1 tissue revealed an increase in number and size of submucosal vessels, confirmed by their frequent inclusion of red blood cells (RBCs; Figure 4C,D; supplementary Figure 1, data not shown; see Figure 7 below). Vessel counts in hematoxylin- and eosin-stained sections showed that vilVEGF1 mice had 3 $\times$ the number of submucosal vessels as WT mice ($P < 0.001$, data not shown), often of larger internal diameter. The transgenic mice also showed a significant increase in pockets of RBCs within the mucosa (Figure 3, $P < 0.001$). Surprisingly, however, vilVEGF1 mice did not develop more or larger mucosal vessels than WT mice.

Hyperproliferation of intestinal epithelium

The thickened mucosa and ectopic epithelium in vilVEGF mice suggested a hyperproliferative phenotype. To further assess this notion, we stained WT and vilVEGF1 tissues for Ki67, a robust marker of proliferating cells in the gastrointestinal tract (21). Restricting our scoring to crypts that were not structurally distorted by cysts (the great majority), we observed a 50% increase in the proliferative zone of vilVEGF1 jejunum, measured by the length of the column of Ki67-positive cells along the crypt-villous axis (supplementary Figure 3, $P < 0.001$). As a second assay of proliferation, mice were injected with bromodeoxyuridine (BrdU) 4 hours before sacrifice, and the length of column of BrdU-labeled cells was measured in jejunal and colon sections. BrdU incorporation was 30% greater in both intestinal segments of transgenic mice (supplementary Figure 2, Figure 5; WT versus vilVEGF1 comparison for each tissue

segment $P < 0.001$). Evidence for increased proliferation of the intestinal epithelium was also obtained in *vilVEGF2* mice (data not shown). Epithelial cells lining cysts incorporated BrdU toward the cyst base, confirming that this epithelium was also proliferative (data not shown). We conclude that VEGF augments proliferation of the intestinal epithelium.

Theoretically, the mucosal hypercellularity might additionally be due to decreased migration of cells toward the lumen and/or decreased cell death. To assess migration, we examined sections of mucosa from mice sacrificed 2 days after pulse BrdU labeling. The distance of travel of pulse BrdU-labeled cells in the transgenic mice was proportional to the increased proliferative index (data not shown), suggesting that decreased migration was not a major factor. Cyst epithelium showed comparable rates of migration from the crypt base (data not shown). Staining for apoptosis markers did not reveal a clear difference between WT and *vilVEGF1* mucosa (data not shown).

Stimulation of intestinal neoplasia

To assay effects of VEGF on intestinal neoplasia, we bred *vilVEGF1* and Min (Multiple intestinal neoplasm) mice. Min mice harbor a mutation in the Adenomatous polyposis coli (APC) gene, the most commonly mutated gene in human colon cancer and a 'gate-keeping' gene for adenoma formation. Recent data revealing numerous microscopic adenomas in small intestines of human APC have underscored the similarity between this condition and the Min model (22). We confirmed that *vilVEGF1*:Min mice achieved higher intestinal VEGF mRNA levels than non-transgenic Min mice (data not shown). *vilVEGF1*:Min and wt:Min littermates were sacrificed at 4 1/2 months and all tumors visible under a dissecting microscope (greater than ca. 1 mm in diameter) were scored by an observer blinded to the genotypes. *vilVEGF1* mice displayed a striking 6-fold increase in intestinal tumorigenesis (Figure 6A; $P < 0.001$ for each intestinal region). Tumors of all sizes were increased in *vilVEGF1* mice (data not shown), with only tumors greater than 4 mm showing a trend toward a disproportionate increase (17 in 12 *vilVEGF* mice versus 1 in 10 non-transgenic mice). Preliminary observations have revealed an increase in intestinal tumorigenesis in *vilVEGF2*:Min mice (data not shown), confirming that this phenotype is caused by the transgene rather than a unique integration event. Therefore, whereas VEGF may particularly stimulate growth of the largest tumors, growth of small tumors was also fostered. Histological analysis of randomly selected tumors confirmed that the vast majority were adenomas (Figure 7, data not shown). Staining of pulse BrdU incorporation showed a higher proliferative index in *vilVEGF1* than WT tumors (mean of 39% BrdU-positive cells in 17 microscope fields from 3 *vilVEGF1* mice versus 25% in 18 fields from 5 WT mice, $P < 0.02$). We conclude that VEGF stimulates intestinal tumorigenesis in the Min model.

These findings were extended by another method. To examine microscopic neoplasia and to exclude the possibility that mucosal thickening in *vilVEGF* mice might have increased ascertainment of tumors, we scored adenomas in random intestinal sections using a 10 $\times$ objective. This assay confirmed the marked increase in macro-adenomas in *vilVEGF1* mice and revealed a similar increase in micro-adenomas (defined as less than 3 villi or crypts in diameter; Figure 6B; $P < 0.001$). Real-time RT-PCR confirmed that VEGF mRNA levels were higher in *vilVEGF1* than non-transgenic Min tumors (data not shown). We conclude that transgenic VEGF stimulates intestinal neoplasia of diverse sizes, including microscopic lesions.

We noted increased features of carcinoma in situ in *vilVEGF1* mice, including loss of differentiation (Figure 7A), desmoplasia (Figure 7B) and pockets of necrosis (data not shown). 5/23 or 22% of WT tumors showed one or more of these features compared to 30 of 68 or 44% of *vilVEGF1* tumors ($P = 0.04$). E-cadherin staining was reduced, particularly at the plasma membrane, in tumor areas with loss of differentiation (Figure 7C). Some adenomas (a minority) in *vilVEGF1*-Min mice displayed cysts (Figure 7D). Moreover, we found 4 polycystic masses

in the vilVEGF1-Min mice, the largest of which was 4 mm in diameter and encompassed more than 100 cysts (Figure 7E, data not shown) and foci of adenoma. Such polycystic masses were also seen in vilVEGF2:Min mice (data not shown), suggesting that some cysts either become neoplastic or can be formed by neoplasms. As in non-neoplastic mucosa, vilVEGF1-Min tumors did not generally display many vessels, by hematoxylin and eosin or vWF staining, though scattered endothelial cells could be detected (Figure 7F, data not shown). vWF staining of the desmoplastic tumors did show increased vascularity (data not shown), the only tumors to clearly do so. We conclude that VEGF stimulates histological progression of larger adenomas and that VEGF-driven cysts can be neoplastic in Min mice.

Direct stimulation of human colonocyte proliferation by endothelial cells

These results suggested that VEGF might augment proliferation of intestinal epithelial cells in part through recruitment of endothelial cells and fibroblasts, independent of angiogenesis or blood flow. To test the concept that interaction with such cells might stimulate proliferation of human intestinal epithelial cells, we generated organotypic cultures. Intestinal epithelial cells are notoriously difficult to culture. We obtained human colon epithelial cells (colonocytes) that were derived at our institution by primary culture of non-tumorous tissue of a patient with APC (M. Clapper, H. Cooper, and K. Campbell, manuscript in preparation). These colonocytes are non-malignant by several criteria, including lack of growth in soft agar, but have sustained bi-allelic APC inactivation, as observed in most human and Min adenomas (23). The colonocytes were cultured on a type 1 collagen matrix, modeled after a method used successfully in short-term culture of human fetal colonocytes (15). Co-cultures were generated with or without colon fibroblasts derived from the same patient and cells of a well-characterized endothelial cell line, MS-1. MS-1 cells grow well in culture, carry functional VEGF receptors, and respond to paracrine signals (24). Co-culture with fibroblasts alone had little impact on the colonocytes, but culture with fibroblasts and endothelial cells (Figure 8) (or endothelial cells alone [data not shown]) stimulated colonocyte proliferation ($P < 0.002$). These results provide proof of principle that endothelial cells, the primary cell type recruited by VEGF, can augment proliferation of APC-mutant colonocytes, independent of blood flow.

Discussion

To better define the biological role of VEGF in intestinal neoplasia, we generated transgenic mouse lines with augmented VEGF production from intestinal epithelium. We found that VEGF has potent effects in normal mucosa, inducing a thickened, hypercellular mucosa with recruitment of endothelial cells and myofibroblasts and augmented proliferation of the epithelium. Moreover, neoplasia of all stages was stimulated. The increase in microscopic neoplasia, well below the size of the typical angiogenic switch, suggests relatively direct effects of VEGF. For example, VEGF may foster proliferation in part through paracrine-stimulated supportive interactions between VEGF-secreting epithelium and recruited endothelial cells and fibroblasts. Proof of principle that proximity to endothelial cells *per se* can stimulate human colonocyte proliferation was obtained from organotypic cultures.

The results of our transgenic studies argue for a tumor-promoting role for the increased VEGF levels observed in sporadic human and mouse colon tumors (2,3,19). Transgenic VEGF overexpression in skin and breast has also accelerated tumor development (13,25). Conversely, targeted deletion of the VEGF gene in lung reduces lung epithelial cell proliferation during development (26). Histopathological features overlapping those observed in vilVEGF mice are seen in some constitutional proliferative diseases of the gut. Polycystic kidney disease cysts afflict kidney and liver. The epithelium of cyst walls is proliferative and associated prominently with vessels and VEGF expression (27), suggesting that VEGF may play a role. We did not observe kidney cysts in vilVEGF mice, but VEGF expression in this tissue may not have been

high enough to yield a phenotype or may not be sufficient, at any level. Both vilVEGF-Min mice and humans with Juvenile Polyposis (JP) display intestinal tumors with cysts and prominent stroma. Deregulated BMP1-SMAD signaling underlies at least some JP (28-31). Evidence has been obtained that the BMP pathway may directly regulate VEGF transcription (32), suggesting that increased VEGF expression may occur in JP and experimental settings of compromised BMP signaling, contributing to their pathology. Mutation of smooth muscle actin causes a cystic expansion of intestinal epithelium in zebra fish (33), underscoring the potential role for mesenchymal cells in such phenotypes.

VEGF receptors (e.g. Flk-1/KDR) are preferentially expressed on endothelial cells, where they act through paracrine and autocrine routes (34). VEGF receptors have also been identified on some malignant cells (35,36). In these cases, it has generally been assumed that an autocrine loop developed through genetic and/or epigenetic instability. We have not ruled out autocrine stimulation of intestinal epithelium by VEGF. However, the recruitment of endothelial cells and fibroblasts by VEGF in vivo and the ability of these cell types to stimulate proliferation of colonocytes in vitro are consistent with paracrine effects. Although emerging evidence indicates that truncating mutations in APC confer some genetic instability in early neoplastic and pre-neoplastic cells (37,38), the proliferative stimulus mediated by VEGF in early neoplastic lesions and non-neoplastic tissue suggests function through normal pathways. Direct supportive interactions between gut epithelial cells and endothelial cells have been described in developing liver and pancreas and adult liver (39-41).

Stimulation of intestinal neoplasia by VEGF suggests an oncogenic role in this tissue and validates VEGF as a target of tumor suppression by p16 (11,12). Both the vilVEGF1-Min and p16-null-Min phenotypes show an increase in colon tumors and tumors with advanced features. A difference is the stronger stimulation of early neoplasia in the vilVEGF1-Min mice. A stronger increase in VEGF in early neoplasia of vilVEGF mice and/or differences in genetic background could contribute. Augmented VEGF was not sufficient to increase vascularity of vilVEGF1-Min tumors yet was still oncogenic.

It is unclear why only some crypts form cysts. Different expression levels of VEGF might be one factor. The cysts generally show greater investment with mesenchymal cells than non-cystic epithelia. These mesenchymal cells may alter epithelial cell fate through paracrine signaling and/or exert mechanical effects that inhibit epithelial cell migration to the lumen. Whereas the mesenchymal cells surrounding cysts express endothelial markers CD31 and CD34, fewer stained for vWF than those lining vessels. Contact with blood might shape differentiation of the vascular endothelial cells, while reciprocal interactions with epithelial cells may modify the differentiation program. Some endothelial cells may be locally derived, others recruited from the bone marrow (42).

Cells of the cyst interior appear to be derived in part by delamination of cells lining the wall opposite the crypt. This pattern may reflect the normal developmental program of intestinal epithelial cells, as many are ultimately shed from the surface into the intestinal lumen. Alternatively, some cysts appear to be open on the end opposite the crypt, with invasion of mesenchymal cells. Cyst epithelial cells of the far wall show evidence for loss of epithelial differentiation, in the form of reduced expression of E-cadherin and claudin-7. Preliminary data showing a lack of distinct staining for vimentin (data not shown) do not support a mesenchymal transition (43,44). The loss of differentiation in cysts may reflect local environmental influences, such as mechanical forces. On the other hand, similar changes were observed in some tumors of vilVEGF1-Min mice and may reflect an additional oncogenic effect of VEGF.

Our results have implications for VEGF-targeted therapy by directly demonstrating that VEGF drives growth of intestinal neoplasia. Moreover, VEGF's effects are evidently not confined to large tumors that require angiogenesis, because neoplasia of all sizes was stimulated. Given that normal intestine is highly vascular, it seems unlikely that oxygenation is limiting for formation and growth of microadenomas, although a role for enhanced delivery of oxygen, cells, and/or serum-borne factors from the submucosa is not excluded. Our results dovetail well with evidence that treatment of Min mice with VEGF receptor antagonists can reduce tumor burden (45,46) and recent results from conditional deletion of the VEGF gene and administration of anti-VEGF antibodies (19). These latter interventions blunted growth of tumors 1 mm or larger in diameter. Korsisaari et al. inferred that the angiogenic switch occurs earlier than generally thought in intestinal tumors. Our findings extend these studies by showing that VEGF stimulates growth of microadenomas. Our interpretation is also different, in that we suspect that angiogenesis is not wholly responsible. Similarly, VEGF may augment growth of micrometastases in humans. These findings underscore that VEGF is a potent mediator of intestinal tissue growth, through pathways inherent to the involved cell types. The limited efficacy thus far of single agent anti-VEGF therapy may reflect technical obstacles to complete inactivation of the pathway and/or redundant signaling in tumors. Our results imply a potential role for anti-VEGF therapy against different stages of intestinal neoplasia, including microscopic lesions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations in this paper

APC	adenomatous polyposis coli
Min	Multiple intestinal neoplasia
RBCs	red blood cells
VEGF	Vascular endothelial growth factor

Vil	villin
vWF	von Willebrand's Factor

Figure 1. Increased red color of viVEGF2 small intestines

Representative WT (L) and viVEGF2 (R) intestines. Note the distinct red color and prominent vessels of viVEGF2 intestines in situ (above) or dissected free.

Figure 2. Increased VEGF expression in vilVEGF intestines

(A) Relative VEGF mRNA levels assayed by real time RT-PCR in designated whole intestinal segments from 3 WT, 3 vilVEGF1, and 1 vilVEGF2 mice. Column: mean value of mice of designated genotype + standard deviation (each reaction in duplicate, WT colon set to 100 arbitrary units). (B) VEGF levels in recombinant standards or mucosal scrapings (5 μ g) from 4 WT, 4 vilVEGF1, and 2 vilVEGF2 mice assayed by ELISA. Column: mean + range of duplicate determinations for one mouse.

Figure 3. Intestinal mucosal thickening and cyst formation in vilVEGF mice

Hematoxylin- and eosin-stained vilVEGF1 and vilVEGF2 jejunal sections show mucosal thickening, cysts, fusion of villi, and increased RBCs compared to WT tissue.

Figure 4. vilVEGF cysts display an inner epithelial lining surrounded by endothelial cells and myofibroblasts

(A, B) Cysts are formed by epithelial cells emerging from crypts. Brown: IHC for Claudin 7. B: dividing (or fusing) cyst. (C-E) Cysts surrounded by CD34-positive cells (D, E; brown), which are largely confined below crypt bases in WT tissue (C). (F) Co-IF staining: α -SMA-positive myofibroblasts (red) are distinct from CD34-positive cells (green; note lack of yellow).

Figure 5. Increased proliferation of vilVEGF1 epithelium

Relative lengths of columns of BrdU-labeled epithelial cells in random jejunum and colon sections of WT and vilVEGF1 littermates. Each graph: totals from 28-49 well-oriented microscopic fields of the designated tissue region from 3-5 mice per genotype (x-axis: BrdU column length (arbitrary units), y-axis: number of BrdU columns of a given length).

Figure 6. Marked stimulation of intestinal neoplasia in vilVEGF1-Min mice

(A) Tumor number in each intestinal section counted under a dissecting microscope from 10 WT-Min mice (L) and 12 vilVEGF1-Min mice (R). (B) Random 'Swiss roll' sections of mucosa from 4 WT-Min (L) and 5 vilVEGF1-Min mice (R) scored under a 10× objective for number of macro- (grey bars) and micro-adenomas (black bars; defined as < 3 villi or crypts in width [see inserted images]).

Figure 7. Advanced histological features in vilVEGF1-Min tumors

(A) Tumor (Tu) with area of undifferentiated cells, characterized by loss of growth in sheets (left of solid line). Right: non-tumor cells. Below dashed line: more differentiated tumor cells. (B) Desmoplastic tumor. Note dense fibrosis and wide separation of neoplastic epithelial sheets. (C) E-cadherin staining is reduced in tumor areas with loss of sheet-like growth (above dashed line). (D) A single villous filled with an adenoma and cyst. Brown: Ki67 staining. (E) Portion of a polycystic mass encompassing more than 100 cysts. Brown: Ki67 staining. (F) vWF staining reveals increased vessels in submucosa (arrows) but not tumor. Arrowheads: scattered vWF-positive cells in tumors.

Figure 8. Stimulation of proliferation of APC-mutant human colonocytes by organotypic co-culture with endothelial cells and fibroblasts

(A) Ki67 staining of human APC-mutant colonocytes ('Epith cells') co-cultured with colon fibroblasts alone (above) or with MS1 endothelial cells ('Endo cells'; below) in the matrix.

(B) Graph: means $\pm$ SD of Ki67 staining of epithelial cells from 4 experiments.