



Aberrant, ectopic expression of VEGF and VEGF receptors 1 and 2 in malignant colonic epithelial cells. Implications for these cells growth via an autocrine mechanism



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ABSTRACT

Vascular endothelial growth factor A (referred to as VEGF) is implicated in colon cancer growth. Currently, the main accepted mechanism by which VEGF promotes colon cancer growth is via the stimulation of angiogenesis, which was originally postulated by late Judah Folkman. However, the cellular source of VEGF in colon cancer tissue; and, the expression of VEGF and its receptors VEGF-R1 and VEGF-R2 in colon cancer cells are not fully known and are subjects of controversy.

Material and methods: We examined and quantified expression of VEGF, VEGF-R1 and VEGF-R2 in three different human colonic tissue arrays containing sections of adenocarcinoma ($n = 43$) and normal mucosa ($n = 41$). In human colon cancer cell lines HCT116 and HT29 and normal colon cell lines NCM356 and NCM460, we examined expression of VEGF, VEGF-R1 and VEGF-R2 mRNA and protein, VEGF production and secretion into the culture medium; and, the effect of a potent, selective inhibitor of VEGF receptors, AL-993, on cell proliferation.

Results: Human colorectal cancer specimens had strong expression of VEGF in cancer cells and also expressed VEGF-R1 and VEGF-R2. *In vitro* studies showed that human colon cancer cell lines, HCT116 and HT29, but not normal colonic cell lines, express VEGF, VEGF-R1 and VEGF-R2 and secrete VEGF into the medium up to a concentration 2000 pg/ml within 48 h. Furthermore, we showed that inhibition of VEGF receptors using a specific VEGF-R inhibitor significantly reduced proliferation (by >50%) of cultured colon cancer cell lines.

Conclusions: Our findings support the contention that VEGF generated by colon cancer cells stimulates their growth directly through an autocrine mechanism that is independent of its primary function in the induction of angiogenesis.

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1. Introduction

Colorectal cancer (CRC) is the fourth leading cause of cancer-related deaths in the world. In the United States it is the third most commonly diagnosed cancer and is the second leading cause of cancer-related deaths. The growth and progression of colon polyps

and cancers, as well as other solid tumors, requires angiogenesis – the formation of new blood vessels from pre-existing vessels [1,2]. Vascular endothelial growth factor A (referred here to as VEGF), identified, cloned and named by N. Ferrara [3,4], is one of the most potent factors that promote tumor angiogenesis [1,2,5–8]. VEGF triggers angiogenesis by inducing endothelial cell growth, migration, and tube formation [5,9].

VEGF is expressed in various cells including myofibroblasts, fibroblasts and endothelial cells in normal tissues, and plays a role in the physiological angiogenesis such as that required for tissue injury healing [1,9,10]. VEGF is also highly expressed by most human cancers (e.g., breast, ovarian, colon, pancreatic and esophageal cancer) and its expression often correlates with tumor progression and poor prognosis [11–17]. Several studies have demonstrated the critical requirement of VEGF for tumor angiogenesis and growth [18–20]. Kondo et al., demonstrated that xenografts of a colon cancer cell line that was stably transfected with the VEGF

Abbreviations: CRC, colorectal cancer; °C, degrees centigrade; h, hours; HCT116, colon cancer cell line; HT29, colon cancer cell line; μ M, micro molar; μ l, micro liter; mm, millimeter; mM, millimolar; mRNA, messenger ribonucleic acid; nm, nanometer; NCM356, normal colonic mucosal epithelial cells; NCM460, normal colonic mucosal epithelial cells; RT-PCR, reverse transcription and polymerase chain reaction; VEGF, vascular endothelial growth factor; VEGF-R1, vascular endothelial growth factor receptor 1; VEGF-R2, vascular endothelial growth factor receptor 2.

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gene formed faster-growing tumors with increased vascularity compared to xenografts of cells transfected with a control vector [18]. Furthermore, Yuan et al., showed that treatment with VEGF neutralizing antibody reduced vascular permeability, vessel diameter and vessel volume in xenografts of human colon adenocarcinoma cells in SCID mice [19]. The first anti-VEGF drug, bevacizumab (a humanized monoclonal antibody against VEGF) was approved for colon cancer treatment in 2004 [20]. It binds directly to VEGF to form a protein complex, which is incapable of binding to VEGF receptor sites. Bevacizumab blocks the growth of new blood vessels to tumors and can extend survival in some patients with metastatic colorectal cancer [20].

All of these studies support the role of VEGF in the induction of tumor angiogenesis that promotes tumor growth by enhanced vascularization. However, the extent to which VEGF can directly affect cancer cell growth independently of its angiogenic properties is not fully known. Furthermore, the presence of VEGF and its receptors in colon cancer cells has not been clearly described and remains controversial. A study of 131 colonic adenocarcinoma patients showed that in 86 patients (66%), the tumors did not express VEGF [21]. Another study of 259 formalin-fixed and paraffin-embedded specimens from sequential patients with colorectal cancer showed that 63.3% (138 of 259) of the specimens did not express VEGF in the tumor cells [22]. Warren et al., examined 30 metastases of human colorectal cancer to liver in a mouse model and showed that VEGF-R1 and VEGF-R2 mRNA expression was limited predominantly to endothelial cells of the tumors [23]. Calvani et al., suggested that different colon cancer cells can differentially express VEGFR-2 [24]. These studies taken together indicate that presence of VEGF and its receptors in colon cancer cells has not been established conclusively, and constitutes a significant gap in our knowledge of colon cancer.

Our present study examined the expression of VEGF and its receptors in human colon cancer specimens – normal and colon cancer tissue arrays; and, in cultured human colon cancer cell lines. We present evidence that: (a) normal colonic epithelial cells do not generate VEGF or express its receptors; (b) VEGF and its receptors are expressed in colon cancer cells; and furthermore VEGF directly promotes colon cancer cell proliferation independently of its primary role as a pro-angiogenic factor.

2. Materials and methods

2.1. Tissue microarray

The use of de-identified commercial human colonic mucosal biopsy specimens for immunostaining was approved by the Institutional review Board at Veterans Affairs Long Beach Healthcare System. Three colon tissue microarrays (TMA; CC05-02-001, CS05-01-001-035 and NC05-01) containing de-identified human colon adenocarcinoma and normal tissue specimens were purchased from Cybrdi Inc., Rockville, MD. CC05-02-001 TMA has 18 matched pairs of human colon adenocarcinomas, tumor margin area and normal colon tissues. CS05-01-001-035 TMA has 22 adenocarcinoma and 3 normal colon specimens. NC05-01 TMA has 3 adenocarcinoma and 20 normal colon specimens.

2.2. Cell culture

Two human colorectal cancer (CRC) cell lines – HCT116 and HT29 (American Type Culture Collection, Manassas, VA) were used. As controls, 2 normal human colonic epithelial cell lines, NCM356 and NCM460 (INCELL Corporation, San Antonio, TX) were used. NCM356 and NCM460 cell lines were obtained by a materials transfer agreement with INCELL Corporation, San Antonio, TX. Cells

were cultured in recommended growth media and cultures were synchronized by serum starving for 18 h prior to all experiments.

2.3. Immunostaining for VEGF and its receptors

Tissue microarrays of paraffin-embedded sections were deparaffinized and immunostained with specific antibodies for VEGF (sc-507, 1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA), VEGF-R1 (sc-316, 1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and VEGF-R2 (sc-504, 1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and visualized using Alexa Fluor dye 568 anti-rabbit secondary antibody (Invitrogen, Carlsbad, CA, USA) using similar methods as described in our previous study [25]. Immunofluorescence was evaluated independently by two investigators (blinded to the microarray coding scheme) using a Nikon Optiphot microscope with epifluorescence. The expression of VEGF, VEGF-R1 and VEGF-R2 in colon cancer cells was graded on a scale from 1+ to 3+. In addition, five fields were randomly selected and staining signal intensity was quantified using MetaMorph 7.0 (Molecular Devices, Downingtown, PA, USA). The expression and localization of VEGF and its receptors in cultured human normal colonic mucosal epithelial cells and in human colorectal cell lines was examined by immunostaining using the above listed antibodies for VEGF, VEGF-R1 and VEGF-R2, Alexa Fluor dye 488 anti-rabbit secondary antibody and methods described previously [26]. The staining signal intensity was quantified using MetaMorph 7.0 (Molecular Devices, Downingtown, PA, USA) and expressed as arbitrary units.

2.4. Determination of VEGF secreted by CRC cells into culture media using enzyme-linked immunosorbent assay (ELISA)

CRC cell lines and normal colonic epithelial cells were cultured in equal density in 12-well plates in complete growth medium overnight. Wells were washed with PBS and cells were cultured in reduced (1%) serum growth media for 6, 24 and 48 h. Cell culture media was removed at each time point, cell debris were removed by centrifugation and cell culture supernatants were frozen for use in the ELISA. The concentration of VEGF in the cell culture supernatants was measured using the human VEGF immunoassay Quantikine ELISA kit (R&D systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Briefly, 200 µl of culture supernatants were added to the wells of a microplate pre-coated with VEGF antibody and incubated for 2 h at room temperature. The plates were then incubated with anti-human VEGF–HRP conjugate at room temperature for 2 h. A color reaction was induced by the addition of premixed TMB substrate solution and was stopped 30 min later by the addition of a stop solution. The absorbance was measured on an ELISA plate reader at 450 nm.

2.5. Determination of VEGF mRNA by reverse transcription real-time quantitative polymerase chain reaction (PCR)

Total cellular RNA was isolated from normal colonic mucosal epithelial cells and CRC cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA (1 µg) was treated with deoxyribonuclease I and reverse transcribed using the GeneAmp RNA-PCR kit (Applied Biosystems, Foster City, CA, USA) as described in our previous studies [27,28]. The mRNA levels of VEGF and β -actin were quantified by real-time PCR using pre-validated QuantiTect assays (Qiagen, Valencia, CA, USA) and the iCycler real-time PCR detection system (Bio-Rad, Hercules, CA, USA). Relative mRNA levels were calculated using the $2^{-\Delta\Delta Ct}$ method and β -actin was used as a reference.

2.6. Determination of VEGF protein by western blot analysis

Expression of VEGF was determined by western blotting using methods described in our previous studies [26,29]. Total cellular proteins were isolated from cultured cells in ice-cold radio-immuno-precipitation assay (RIPA) buffer. Protein concentration was determined by standard Bradford assay (Bio-Rad, Hercules, CA, USA). Proteins were subjected to SDS-PAGE and transferred onto a nitrocellulose membrane. The membranes were incubated with specific antibodies against VEGF (1:250, Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by peroxidase-conjugated secondary antibodies. Immunoreactive proteins were visualized using ECL detection system. β -actin was used as an internal control reference.

2.7. Cell proliferation assay

CRC cells were cultured on glass coverslips and serum starved for 18 h. CRC cell proliferation at baseline was determined by Bromodeoxyuridine (BrdU) assay using the BrdU Cell Proliferation Kit (EMD Millipore Corporation, Billerica, MA) according to the manufacturer's instructions. The total cell count and number of BrdU positive cells were determined. Cell proliferation was expressed as percent of BrdU positive cells.

2.8. Statistical analysis

Data are presented as mean \pm SD. Statistical significance was analyzed by either Student's *T*-test or analysis of

variance. A *p* value less than 0.05 was considered statistically significant.

3. Results

3.1. Increased protein expression of VEGF and its receptors VEGF-R1 and VEGF-R2 in human CRC tissue specimens

Immunofluorescence staining for VEGF and its receptors VEGF-R1 and VEGF-R2 was performed in 43 histological specimens of human colorectal cancer (CRC) and 41 normal colonic mucosa. Expression of VEGF, VEGF-R1 and VEGF-R2 was strong (aberrant) in CRC tissues compared to normal colonic mucosa (Fig. 1). VEGF, VEGF-R1 and VEGF-R2 was localized to CRC cells in CRC tissues (Fig. 1). Normal colonic mucosa specimens had only minimal staining (comparable to background) for VEGF and its receptors. All human colon cancer tissue specimens stained positive for VEGF and its receptors; 63% of array sections had strong staining (2+ or 3+ on a scale from 1+ to 3+) for VEGF and its receptors in malignant cells, while 37% of the array sections had weak staining (1+ on a scale from 1+ to 3+) for VEGF and its receptors in malignant cells.

3.2. CRC cell lines express and secrete VEGF

We examined expression of VEGF mRNA and protein in the human CRC cell lines, HCT116 and HT29, and in normal colonic epithelial NCM356 and NCM460 cells by real time RT-PCR and western blotting. Normal colonic epithelial cell lines expressed

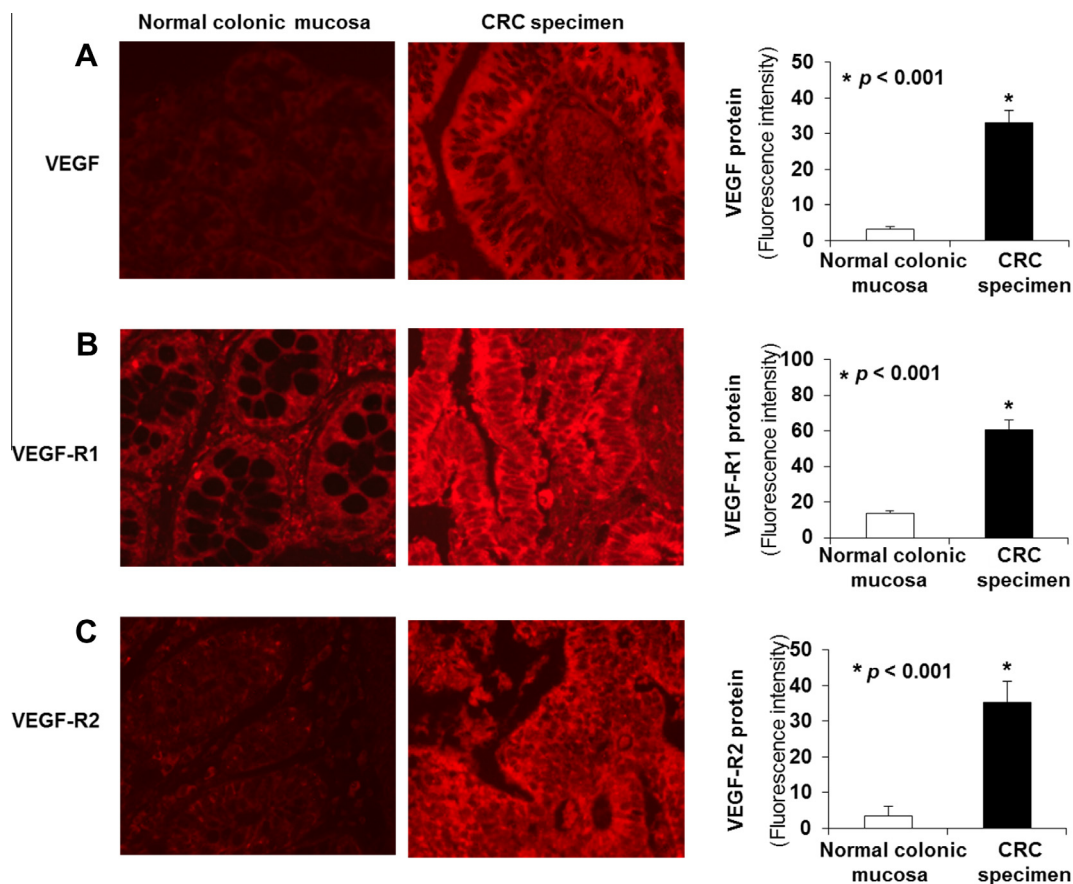


Fig. 1. Immunostaining of VEGF, VEGF-R1 and VEGF-R2 in human colonic specimens A, B and C. The expression of VEGF, VEGF-R1 and VEGF-R2 was examined in colon cancer tissue specimens and compared to normal colonic mucosa by immunostaining. Quantification of the staining intensity was performed for five randomly selected fields using MetaMorph 7.0 (Molecular Devices, Downingtown, PA, USA) and is shown in graphical representation. Colon cancer cells demonstrated significantly increased expression of VEGF, VEGF-R1 and VEGF-R2 (red fluorescence) compared to normal colonic mucosa. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article that presents color images.).

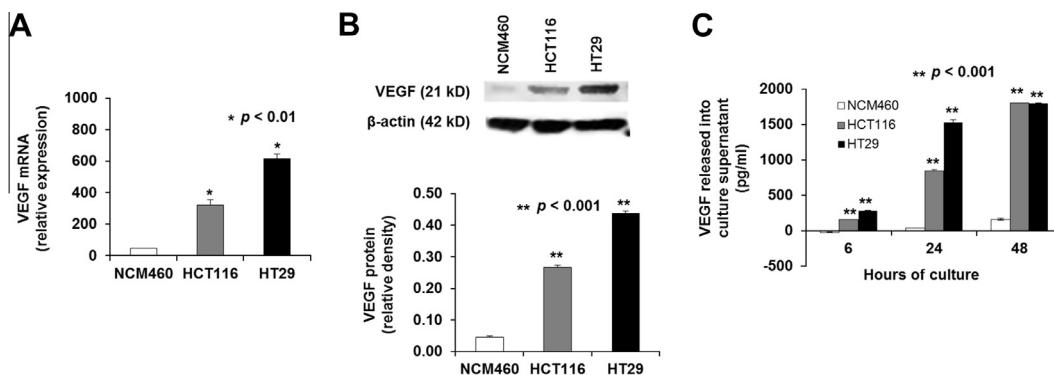


Fig. 2. Expression and secretion of VEGF into culture media by CRC cell lines and normal colonic epithelial cells. (A). Real-Time RT-PCR determination of VEGF gene expression. Values are mean \pm SD of 3 independent experiments. VEGF mRNA expression was 13.2-fold and 7-fold higher (both $p < 0.01$) in HCT116 and HT29 cells, respectively vs. NCM469 cells. (B). Western blotting determination of VEGF protein levels. Values are mean \pm SD of 3 independent experiments. VEGF protein expression was 6-fold and 9.8-fold higher ($p < 0.001$) in HCT116 cells and HT29 cells, respectively, vs. NCM469 cells. (C). ELISA determination of VEGF secreted into culture media by NCM469, HCT116 and HT29 cells. Values are mean \pm SD of 3 independent experiments. Normal colonic epithelial cells do not secrete VEGF in 6 h of culture and only secrete minimal VEGF upon culture for 48 h. HCT116 and HT29 secrete significantly higher levels of VEGF that accumulate and increase in culture media with time.

no or minimal VEGF mRNA and protein. HCT116 and HT29 CRC cells expressed 13.2-fold and 7-fold higher VEGF mRNA, respectively (both $p < 0.01$), vs. normal colonic mucosal epithelial cells (Fig. 2A). These cells also had 6-fold and 9.8-fold higher VEGF protein expression, respectively (both $p < 0.001$), vs. normal colonic mucosal epithelial cells (Fig. 2B).

Next, we quantified the levels of VEGF secreted into culture media by NCM460, HCT116 and HT29 cells over a 48 h period. VEGF was detected in culture medium supernatant of HCT116 cells by ELISA within 6 h and the VEGF levels continued to rise for 48 h (the duration of the study) significantly compared to NCM460, which only secreted very low VEGF amounts, which were undetectable until 24 h of culture (Fig. 2C).

3.3. CRC cells express VEGF-R1 and VEGF-R2

We examined expression of VEGF, VEGF-R1 and VEGF-R2 in HCT116 and HT29, and in normal colonic epithelial NCM460 cells

by immunostaining. Protein expression of VEGF and its receptors, VEGF-R1 and VEGF-R2 was strong in CRC cells vs. absent or minimal in normal colonic mucosal epithelial cells (Fig. 3).

3.4. VEGF is critical for colon cancer cell proliferation in vitro

Since our studies demonstrated that colon cancer cells express VEGF and its receptors, we therefore examined the functional consequence of blocking VEGF receptor kinase activity (VEGF signaling) on colon cancer cells proliferation. We inhibited VEGF receptor kinase using AAL993 (2-(Pyridin-4-ylmethylamino)-N-[3-(trifluoromethyl)phenyl]benzamide), a highly specific and potent inhibitor of VEGF-R1 and VEGF-R2 that blocks the downstream action of VEGF signaling [30]. Cells were treated with 1 μ M AAL993 or solvent (DMSO) for 4 h and the effect of blocking VEGF function on CRC cell proliferation was examined ($n = 3$). Blockade of VEGF signaling using the VEGF receptor kinase inhibitor significantly decreased HCT116 and HT29 cells' proliferation by

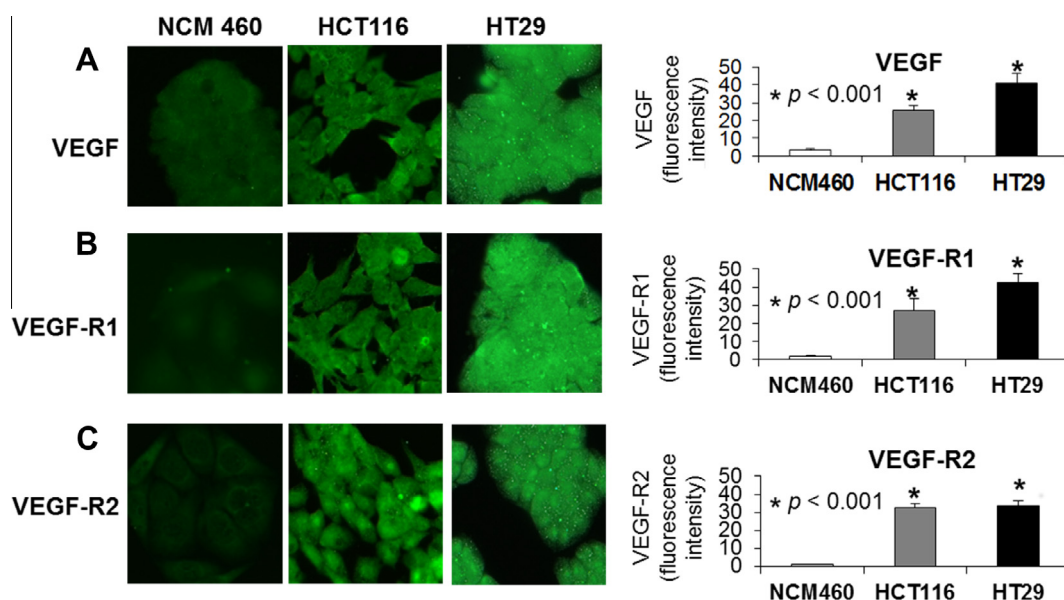


Fig. 3. Immunostaining for VEGF and its receptors in cultured CRC cell lines and normal colonic epithelial cells. A, B and C. The expression of VEGF, VEGF-R1 and VEGF-R2 was examined in HCT116 and HT29 CRC cell lines and compared to normal colonic epithelial NCM460 cells by immunostaining. Values are mean \pm SD of 3 independent experiments. VEGF and its receptors, VEGF-R1 and VEGF-R2 are strongly expressed (green fluorescence) in HCT116 and HT29 cells compared to normal colonic epithelial NCM460 cells. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article that presents color images.)

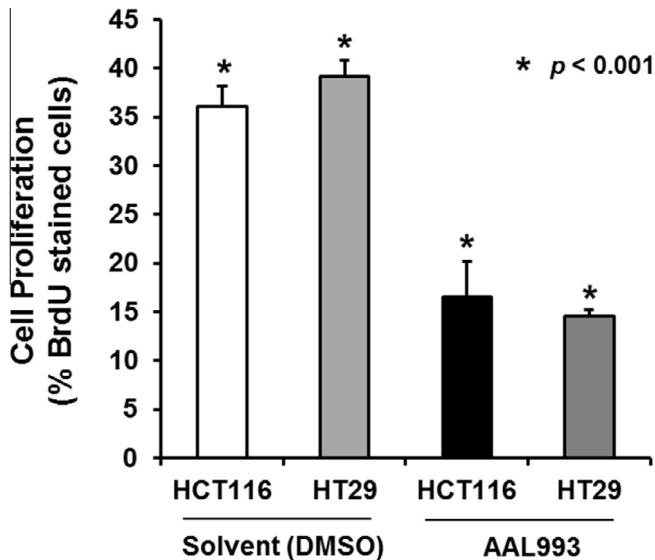


Fig. 4. Inhibition of VEGF-R decreases cell proliferation of CRC cells. Cells were cultured in the presence of VEGF-R inhibitor AAL993 (1 μ M) or solvent (DMSO) for 4 h and cell proliferation was determined by BrdU assay. Cell proliferation was calculated as the percent of total cells that stained positively for BrdU. Values are mean \pm SD of 3 independent experiments. Inhibition of VEGF receptors using AAL-993 significantly decreased proliferation of both HCT116 and HT29.

2.2-fold and 2.7-fold, respectively (both $p < 0.001$) (Fig. 4) indicating that the proliferation of these cells is partly dependent on VEGF.

4. Discussion

VEGF plays a critical role in colon cancer growth via angiogenesis (by promoting endothelial cell growth, migration and survival), which is responsible for vascularizing colon tumors and [9,31,32]. VEGF is produced by various cell-types including myofibroblasts, endothelial cells, macrophages and transformed cells [9]. Once secreted by these cells, VEGF can act on other cells in a paracrine manner and promote cell survival, migration and differentiation. Whether colon cancer cells secrete VEGF and whether VEGF can directly stimulate the growth of these cells has not been conclusively established. Our present study demonstrated that colon cancer cells secrete VEGF and express VEGF-R1 and VEGF-R2, indicating the possibility that VEGF can directly stimulate growth of colon cancer cells via autocrine signaling.

Several studies have demonstrated that normal colonic epithelium does not express VEGF [21,22]. Studies investigating the role of VEGF in colon cancer growth have primarily focused on endothelial cells, and its role in the induction of the angiogenesis responsible for vascularizing colon tumors [22,24,31–33]. However, the concept that VEGF can be generated by colon cancer cells remains controversial. Moreover, whether colon cancer cells express VEGF-R1 and VEGF-R2 has not been conclusively established. Fan et al. demonstrated a weak VEGF-R1 expression in eight of ten primary colon cancer specimens and significantly higher VEGF-R1 expression in six of nine metastases of colon cancer to liver examined [34]. In that study, VEGF-R1 was not detectable in normal colonic mucosa. VEGF-R2 immunostaining in colon cancer tissues was shown to be primarily in endothelial cells, while carcinoma cells stained faintly [22,35]. Calvani et al., suggested that colon cancer cells can differentially express VEGF-R2 based on the different population of the cells within the tumor that they originate from, indicating that there may be a differential responsive of colon cancer cells to VEGF [24].

Our study showed that CRC cells in colon cancer tissues and cultured CRC cell lines express both VEGF-R1 and VEGF-R2. This study also demonstrated that the inhibition of VEGF receptor kinase using specific inhibitor AAL-993 results in decreased cell proliferation of cultured colon cancer cells. Recently, a VEGF receptor tyrosine kinase inhibitor with activity against all three receptors for VEGF – cediranib, has been proposed for the treatment of metastatic colon cancer and has been shown to have a similar activity to bevacizumab [36]. Our current study indicates a role of VEGF in directly mediating proliferation and growth of colon cancer cells that express VEGF-R1 and VEGF-R2 via an autocrine mechanism in addition to the previously recognized role of VEGF as a potent inducer of cancer angiogenesis. This study may have significant implications – assessment of VEGF and its receptor expression in biopsies can provide additional information whether a particular cancer patient will respond to anti-VEGF treatment. Furthermore, VEGF may perhaps regulate autocrine growth of other non-colonic malignant cells in various tissues.

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