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Vascular endothelial growth factor – key mediator of angiogenesis and promising therapeutical target in ulcerative colitis

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Abstract

Ulcerative colitis (UC) is an inflammatory bowel disease, triggered by an inappropriate immune response of colonic mucosa. Angiogenesis is an important part of inflammatory process, enhancing inflammation in a vicious circle that aggravates mucosal damage and remodeling. The most important pathway for angiogenesis in ulcerative colitis involves vascular endothelial growth factor (VEGF) and endoglin (CD105) and can be used as target for adjuvant therapy in order to improve patients' outcome. We present a retrospective cohort study evaluating mucosal expression of VEGF and CD105 and their correlation with patients' evolution and risk of relapse. In our study, patients with UC have correlated increases of VEGF expression and microvessel density (evaluated with CD105 staining), sustaining the hypothesis that angiogenesis is not just a passive process driven by inflammation, but an active player of mucosal lesions in ulcerative colitis.

Keywords: ulcerative colitis, angiogenesis, VEGF, endoglin, oxidative stress.

Introduction

Ulcerative colitis (UC) is an inflammatory bowel disease with chronic evolution, marked by repeated flare-ups, despite sustained treatment. Although, the trigger that produces the disease is unknown, inappropriate immune response of colonic mucosa is the main mechanism of initiation and chronicity of inflammation [1].

Angiogenesis is an important part of the pathogenesis of UC, multiple studies demonstrating the significant role that angiogenetic molecules [as vascular endothelial growth factor (VEGF), vascular endothelial growth factor receptor (VEGFR), endoglin (CD105), platelet endothelial cell adhesion molecule-1 (PECAM-1)] are playing in bowel inflammation [1–3]. Increased angiogenesis can be evaluated microscopically, on biopsies, but also *in vivo*, using endoscopy with narrow-band imaging (NBI) [4]. Usually, pathologist is offering little attention to vascular changes in inflammatory bowel disease, since it was considered a passive process, driven by inflammation. Recent studies demonstrated that angiogenesis in UC has two components: an inflammatory angiogenesis triggered and modulated by inflammation and an important process of pathological angiogenesis, active part of the lesion, not inflammatory driven, that needs to be particularly addressed during therapy [1, 3].

Angiogenesis is the multi-step process of developing new vessels from pre-existent endothelial cells. These cells are receiving stimulation, undergoing proliferation, migration and adhesion, in order to form aggregates that will become vascular structures after lumen formation [1]. In UC, angiogenesis is triggered by inflammation, but also by the abnormal immune response. After regeneration, angiogenesis in UC continues, enhancing inflammation in a vicious circle that aggravates mucosal damage and remodeling [1, 3]. Since more and more data are sustaining the importance of vascular remodeling and angiogenesis in mucosal lesions of UC, also a vascular theory for UC pathogenesis emerged [5]. As a result, anti-angiogenetic molecules were tested and proposed as treatment for UC, at least as adjuvant therapy [6, 7].

The most important problem to be yet investigated is the exact role of vascular changes and angiogenesis in pathogeny of UC. Since UC is an inflammatory disease and the trigger for inflammation is bowel mucosa, certainly is expected to have some vascular lesions secondary to the inflammation. These vascular changes are also increasing inflammation and mucosal remodeling [6, 8, 9]. Vascular remodeling can be the result of subsequent ulcerations and healing of the mucosa and it can be triggered by multiple pro-angiogenetic molecules released in inflammatory conditions [10]. This should not be a therapeutic target, since it is sufficient to end the inflammatory cascade in order to correct the vascular lesions [11]. However, in UC, there is, from the early stages, a pathological angiogenetic response, which plays a direct role in mucosal

lesion and healing, triggered by morphological and functional anomalies of VEGF and its tissue receptors [5, 12]. These changes are partially independent of inflammation and can represent a therapeutical target.

The exact pathway of pathological angiogenesis in UC is not fully understood, several hypothesis being examined for validation: immune-driven angiogenesis, CD40–CD40 ligand pathway, angiopoietin 2 signaling pathway [6, 7, 13, 14].

VEGF is, no doubt, the most studied angiogenetic factor in inflammatory bowel diseases (IBDs), since its level in circulating blood and bowel mucosa in elevated in patients with IBD and correlates with the activity of the disease [15, 16]. It produces, in the bowel mucosa, vasodilatation, increased vascular permeability and activation of quiescent endothelial cells triggering angiogenesis [17]. VEGF is, in fact, a family of growth factors, including VEGF-A, -B, -C, -D, -E, and -F that bind to some specific cell receptors (VEGFR-1, -2 and -3) and have various roles in vascular genesis [18, 19]. VEGF-A and its receptors are promoting inflammation, enhancing leukocyte adhesion, worsening and maintaining the inflammatory process [20]. VEGF-B has complementary effects, while the other two factors are stimulating lymph-angiogenesis [19].

During inflammation, VEGF is produced by the mesenchymal cells of the stroma, endothelial cells, activated T-cells, neutrophils, and monocytes [18, 21]. It plays a major role in angiogenetic cascade, along with CD105 (endoglin), a co-receptor for transforming growth factor-beta (TGF-β) superfamily [22]. High levels of circulating and tissue VEGF and CD105 were found in patients with UC and are considered responsible for expansion of microvascular bed of large bowel mucosa through pathological angiogenesis. Also, increased activity of CD105 is producing a late and inappropriate resolution of colonic inflammation [23].

Thus, it seems that in UC, VEGF and endoglin (CD105) play a passive role (increasing vascular permeability in inflammatory conditions), but also an active role (inducing and modulating mucosal remodeling).

Aim

In this paper, we aimed to describe the microvascular changes in the mucosa of patients with UC, as they are revealed by VEGF and CD105 (endoglin) expression.

Patients, Materials and Methods

Our study is part of a much larger cohort study including patients with IBD, study that is trying to identify clinical, serological, endoscopic and histological features with prognostic value in this disease. Among other histological parameters evaluated, it was also VEGF and CD105 expression in biopsies taken from patients with IBD. All procedures were carried in perfect compliance with national and European research laws and professional deontology and study design was approved in 2013 by the Ethics Commission of "Colentina" University Hospital, Bucharest, Romania.

Criteria of inclusion: patients with confirmed UC (according with 2012 *European Crohn's and Colitis Organization Consensus*) that agreed to participate and signed the informed consent. Criteria of exclusion: invasive malignancies during the study, non-compliance to therapy or surveillance schedule. Thus, in this study were included 45 consecutive cases of UC, in order of presentation at the debut of the study. No patient was excluded or abandoned the study during the next 12 months.

After signing an informed consent, patients underwent a through personal and familial anamnesis, a full clinical assessment and an endoscopic evaluation (ileocolonoscopy with NBI and magnification chromoendoscopy). For each patient, clinical and endoscopic scores were calculated, including the Mayo score, the most used score for UC [24]. During colonoscopy, multiple biopsies were taken, using Endokit for a proper orientation, as it follows: one rectal biopsy, 1–3 biopsies from each colon segment, including most inflamed areas as well as less affected mucosa and one biopsy from terminal ileum. Tissue fragments were immediately immersed in 10% buffered formalin. They were automatically processed using a Leica Tissue Processor and a standardized protocol for digestive endoscopic tissue samples: 18–24 hours fixation in 10% buffered formalin, rinse in water, two baths of 30 minutes, dehydration in three baths of 96% ethanol 90 minutes each, then two one-hour baths of absolute ethanol, then clearing with toluene (three baths of one hour each of xylene, and, finally, three one-hour baths of histological paraffin, at 58°C). The specimens were paraffin-embedded and sectioned in 3 μm slices, using a Leica semi-automated microtome. At least two slides from each paraffin block were routinely stained with Hematoxylin–Eosin (HE), examined by two independent fully trained pathologists and a histopatological diagnosis was formulated.

Patients received treatment according to international protocols [25], and they were instructed to return after 12 months. During this period, they had permanent connection with a gastroenterologist. All procedures were repeated after one year (12 months) of follow-up.

Control group was composed from 45 samples of normal colonic mucosa, harvested from surgical samples belonging to patients with tumoral pathology, mucosa taken from at least 20 cm distance from any macroscopic or microscopic lesion. These samples were processed using the same technique.

After histological diagnosis, all tissue samples were evaluated by two-independent fully trained pathologists, considering multiple histological and immunohistochemical features, including VEGF and CD105 expression.

We used a manual tri-stage technique for performing immunohistochemistry (Table 1). Citrate buffer antigen-retrieval from Leica Biosystem was used as pretreatment, as chromogen was used 3,3'-Diaminobenzidine (DAB), as detection kit – Novolink Polymer Detection System from Leica Biosystems and the sections were counter-stained using Hematoxylin. Positive controls were offered by the manufacturer of antibodies (renal tissue for CD105 and tonsil for VEGF).

VEGF expression was evaluated in mucosal lamina propria (stroma, no inflammatory cells), as well as in epithelial cells, where it represents a marker of oxidative stress [26]. Each biopsy received two scores for the VEGF expression: VEGFs (for stroma) and VEGFe

(for epithelium). VEGFs were evaluated on a semiquantitative scale ranging from 0 (negative) to 3 (intensely positive), while for VEGFe it was used the scale proposed by Remmele & Stegner (Table 2) [27].

Table 1 – ***Reagents used for immunohistochemistry***

Antigen	Clone	Manufacturer	Dilution	Other reagents used
VEGF polyclonal antibody	PA5-16754	Thermo Fisher Scientific	1:100	Sodium citrate (pH 6), H_2O_2–methanol, 3% BSA–PBS, HRP-conjugated secondary antibody, DAB.
CD105 polyclonal antibody	PA5-16895	Thermo Fisher Scientific	1:25	Sodium citrate (pH 6), H_2O_2–methanol, 3% BSA–PBS, HRP-conjugated secondary antibody, DAB.

VEGF: Vascular endothelial growth factor; BSA–PBS: Bovine serum albumin–phosphate-buffered saline; HRP: Horseradish peroxidase; DAB: 3,3'-Diaminobenzidine.

Table 2 – ***Classification scoring system***

Percentage of positive cells	Intensity of staining	IRS – points	IRS – classification
0: No positive cells	0: No color reaction	0–1: Negative	0: Negative
1: <10% of positive cells	1: Mild reaction	2–3: Mild	1: Positive, weak expression
2: 10–50% positive cells	2: Moderate reaction	4–8: Moderate	2: Positive, mild expression
3: 51–80% positive cells	3: Intense reaction	9–12: Strongly positive	3: Positive, strong expression
4: >80% positive cells			

IRS: Immunoreactive score.

CD105 expression was evaluated in mucosal lamina propria, using also a score ranging from 0 to 3, calculated as it follows: it was identified the area of maximum vascularization and all positive microvessels were counted in one ×100 field (×10 objective and ×10 ocular, 1.8 mm diameter). There were obtained values between 16 and 214 (mean value 87.5) in the first group and values from 12 to 223 in the second group (mean value 81.5). The control group had values between 4 and 38 (mean value about 19). All cases with microvessel density of maximum 40 were considered similar with the control group and received score 0 for CD105 expression (no angiogenesis). Values between 41 and 100 received score 1, between 101 and 200 – score 2, and beyond 201 – score 3. This method was inspired by Weidner *et al.* method for evaluating angiogenesis in prostate carcinoma [28].

Cases with lack of concordance between the two evaluators were reevaluated with a third, independent pathologist, using a multiple head microscope and the consensus was reached in all cases.

For statistical correlation, there were used Student's *t*-test for two groups and analysis of variance (ANOVA) test for three or more groups.

Results

In our cohort, we included 31 males and 14 women, with a median age of 41.51 years (age ranging between 19 and 72 years old). During this study (12 months), 16 (~35%) patients had an unfavorable evolution (no remission, relapse or UC complications), while 29 (~65%) patients had a favorable outcome (no relapse, no complication, a better clinic and endoscopic status) (Figure 1).

At the moment of inclusion in this study, 31 patients had pancolitis, while nine patients had left colitis and five patients had only proctitis (Figure 2). In the end of the study, all patients with proctitis and left colitis preserved the reduced extension of the disease, while from the group of patients with pancolitis, two patients had only left colitis, the remaining 29 preserving the disease extension from the beginning.

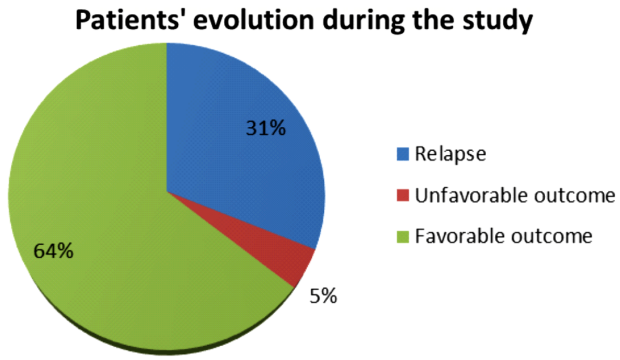

Figure 1 – ***Evolution of patients during the study. Note that relapse was responsible only for a minority of cases with unfavorable evolution (14 out of 45), while most of the patients were included in this group because they did not experience a significant improvement despite adequate treatment.***

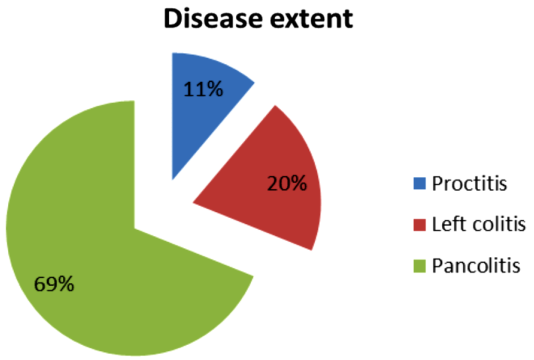

Figure 2 – ***Most patients included in this study had extensive disease (evaluated endoscopically, using Mayo score, and histologically).***

We examined 90 biopsies from UC patients (45 from the beginning of the study – T0 and another 45, from the same patients, after one year – T1). In control samples, VEGFs expression was absent in 16 cases (score 0) or weak in four cases (score 1), while CD105 expression was minimum (there were identified between four and

38 positive structures on a ×100 field). In the cohort of UC patients, VEGFs expression was increased in 12 biopsies (values of 2 and 3), six from T0 and six from T1. Another 48 (53.33%) biopsies received a value of VEGFs of 1 (while in the control group only 20% of samples had VEGFs 1), this result being considered an equivocal increase of VEGFs expression (Figure 3).

From the study group, six patients had unequivocal increase of VEGFs expression at T0. All of them experienced a decrease of VEGFs expression to 1 (equivocal increase). On the other hand, none of the patients with increased VEGFs expression in T1 had previous abnormal expression of VEGF, practically experiencing an increase of angiogenetic activity during the 12 months of the study. Five patients had a favorable clinical and endoscopic evolution at this time, the increase of VEGFs expression correlating with a clinical significant relapse in just one patient. Correlation of VEGFs expression with patients' evolution (clinical and histological) did not achieve statistically significant values (Student's *t*-test, two-tailed, p=0.1667 and p=0.1643, respectively) (Figure 4).

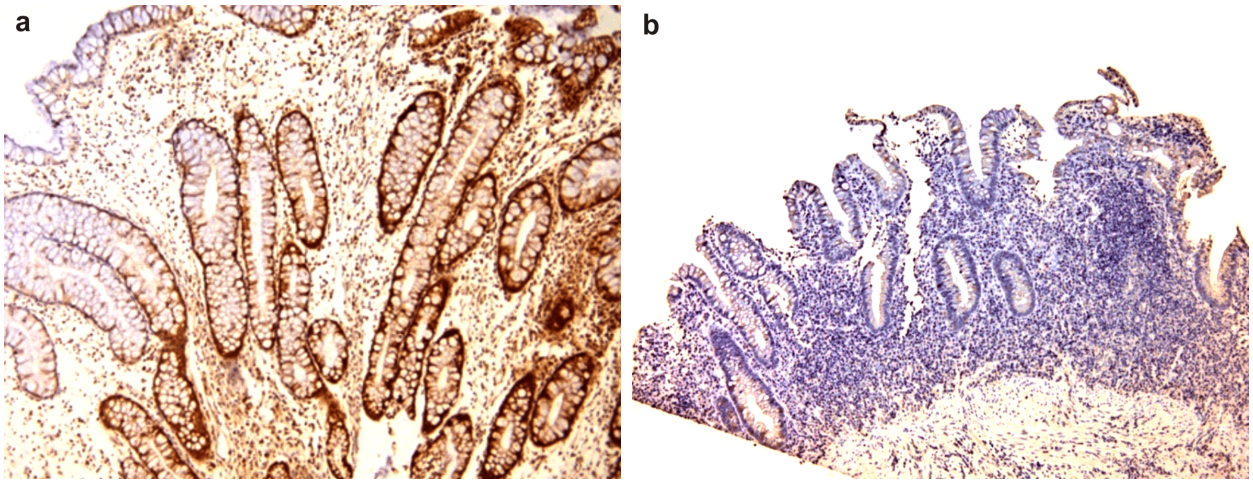

Figure 3 – ***Variation of VEGF expression in a patient with stationary evolution (no relapse but no clinical or endoscopic remission), although he had a slightly increased microscopic inflammatory activity. First biopsy (a) was collected in T0 and the second sample was collected after 12 months (T1). Note the significant decrease of VEGFe (from 3 to 0) and of VEGFs (from 2 to 0). Anti-VEGF antibody immunostaining: (a) ×100; (b) ×40. VEGF: Vascular endothelial growth factor; VEGFe: VEGF for epithelium; VEGFs: VEGF for stroma.***

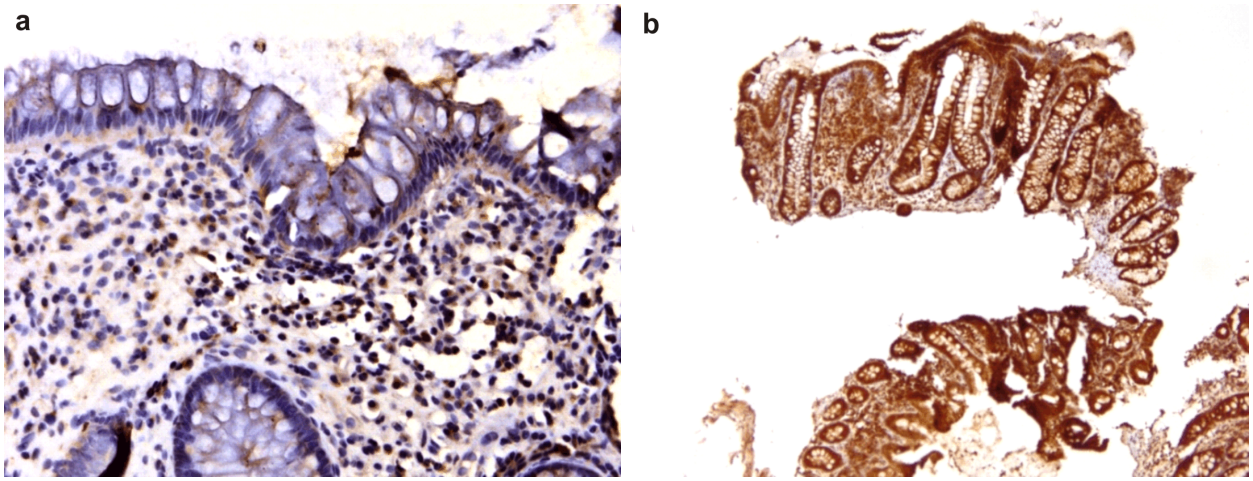

Figure 4 – ***VEGF expression in a patient with UC. First biopsy (a) was taken in T0, when patient had a mildly active disease. During the 12 months of the study, he underwent two relapses and was initiated on biologic therapy. Second biopsy (b) was taken at T1, when from clinical and endoscopic point of view, he was in remission. The first biopsy reveal absence of expression for VEGFe (0) and a very low expression of VEGFs (1), while the second biopsy reveals intense positivity for VEGF in stroma and epithelial cells. Anti-VEGF antibody immunostaining: (a) ×200; (b) ×40. VEGF: Vascular endothelial growth factor; UC: Ulcerative colitis; VEGFe: VEGF for epithelium; VEGFs: VEGF for stroma.***

The only immunohistochemical marker that was significantly correlated with VEGFs was the stromal expression of CD105 (endoglin) (Student's *t*-test, two-tailed, p=0.0139) (Figure 5).

CD105 expression was increased in most of the examined biopsies (75 samples, representing 83%), 39 from T0 and 36 from T1. Eighteen patients underwent an increase of microvessel density, 19 patients a decrease and, in eight cases, the expression of stromal CD105 was constant (Figure 6).

CD105 stromal expression was significantly correlated with patients' evolution (Student's *t*-test, two-tailed,

p=0.0156) and risk of relapse (Student's t-test, two-tailed, p=0.0096).

Although, during the study, the patients received therapy, they underwent an insignificant global decrease of CD105 and VEGFs expression, some of them experiencing even an increase of angiogenetic activity, despite a favorable clinic and endoscopic evolution (Figure 7).

VEGFe expression was increased in study group in 66 biopsies, while in control group it was 0 in all samples. From the study group, 36 patients had increased VEGFe at T0, 24 of them keeping the same value after 12 months. From these 24 patients, 17 (70.83%) had a favorable evolution during these months (no relapse, improvement of clinical and endoscopic status) and 15 (62.5%) had an improvement of histological lesions (lower Geboes score). Three patients had a higher score of VEGFe (correlated in all cases with an unfavorable evolution) and nine patients had a lower VEGFe value (four with favorable clinical evolution and five with poor outcome).

VEGFe did not correlate with VEGFs (Student's t-test, two-tailed, p=0.5078) or with CD105 stromal expression (Student's t-test, two-tailed, p=0.3467).

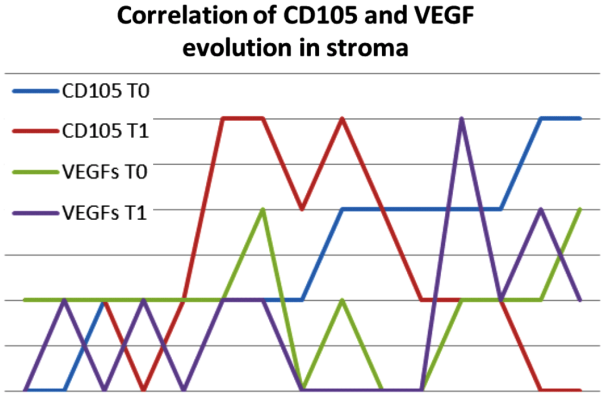

Figure 5 – ***CD105 (endoglin) and VEGFs expression are evolving in the same manner, since they are involved together in angiogenesis. VEGF: Vascular endothelial growth factor; VEGFs: VEGF for stroma.***

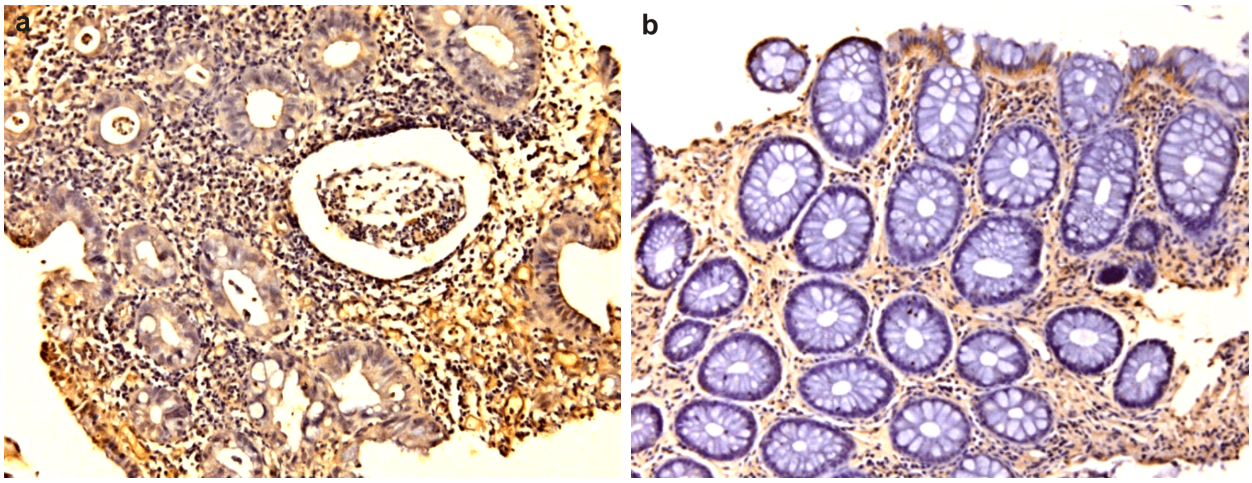

Figure 6 – ***Stromal expression of CD105 (endoglin) in a patient with UC. Note the significant decrease from the first biopsy (a) to the second one (b). The patient had, also, a favorable clinical and endoscopic evolution during this study. Anti-CD105 antibody immunostaining: (a and b) ×100. UC: Ulcerative colitis.***

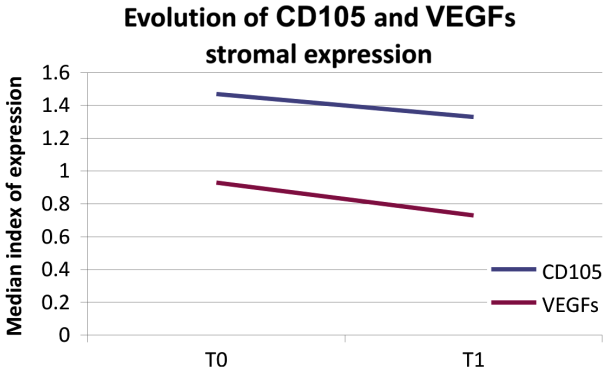

Figure 7 – ***Global evolution of CD105 (endoglin) and VEGFs expression in our study. VEGF: Vascular endothelial growth factor; VEGFs: VEGF for stroma.***

Discussion

Epithelial expression of VEGF is a result of inflammation and effect of oxidative stress on colonic mucosa, mirroring cell distress and possible achievement of DNA lesions, as identified in other studies concerning gastric mucosa [29, 30]. Although our study failed to correlate epithelial expression of VEGF with patients evolution or with other angiogenesis markers, data from literature permit the use of this marker, for research purposes, to stratify patients according to their risk to develop epithelial neoplasia. Since Romania has an increasing prevalence of UC and is facing an increasing number of patients with long-standing disease and higher risk of epithelial malignancies, expression of VEGF in epithelial cells can represent a significant marker for long-time surveillance of these patients. Further studies, on longer periods of time should reveal if epithelial expression of VEGF is a reliable marker to be used as prognosis indicator in IBD. Since there is new information about the fact that anti-tumor necrosis factor (TNF) molecules are interfering with VEGF pathway of angiogenesis and modify the expression of VEGF in relation with mucosal healing, the ultimate goal in IBD treatment [29], the results of our study will be the base for a further study concerning effects of treatment on tissue expression of VEGF.

Stromal expression of CD105 (endoglin), a pro-

angiogenic molecule induced by tissue hipoxia [31–34] and VEGF, in our patients, had a correlated evolution during the study (12 months), confirming that the two molecules are together involved in the same processes. It is important to know that the main pathway for angiogenesis in UC is VEGF–CD105 controlled, since some of the steps of this pathway are possible therapeutical targets. This data are sustaining experimental data from literature [1, 22, 23] and are emphasizing the importance of addressing angiogenesis as an active process in IBD, especially in non-responders to anti-TNF-α therapies [35].

Stromal expression of VEGF (correlated with stromal expression of CD105) is the proof that VEGF-mediated angiogenesis is not just driven by inflammation, but it represents an independent process involved in mucosal remodeling, as other studies suggested [36]. Although, probably, this is not the main inflammatory process, it can represent a target for an adjuvant therapy that can improve patients' response to biological treatment currently in use [36, 37].

Conclusions

In UC, microvascular bed of colonic mucosa is suffering expansion and remodeling driven by inflammatory process (normal angiogenesis), but also undergo a pathological angiogenesis, mediated by the pathway of VEGF and CD105. Anti-inflammatory therapy does not obtain, in our patients, a normalization of stromal pro-angiogenetic activity and fails to interrupt the vicious cycle of inflammation and angiogenesis. This study demonstrates a significant correlation between microvessel density, evaluated using CD105 staining, and VEGF expression, sustaining the hypothesis that VEGF–CD105 controlled angiogenesis is an active player of mucosa damage and remodeling and needs to be addressed with specific adjuvant therapy in order to optimize patients' outcome.

Conflict of interests

The authors declare that they have no conflict of interests.

Author contribution

Radu Bogdan Mateescu and Alexandra Eugenia Bastian equally contributed to this paper.

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