Abstract. Background: Colon cancer (CC) is frequently complicated by thromboembolic episodes. Thrombin plays a role in angiogenesis and among others induces the synthesis of vascular endothelial growth factor (VEGF) and its receptors (VEGFR-1 and VEGFR-2). The aim of this study was to assess the expression of prothrombin fragment F1+2 (F1+2), a byproduct in thrombin generation (indicating the presence of thrombin), in relation to the presence of VEGF:VEGFR-2 (VEGF:VEGFR-2), as an indicator of VEGFR-2 activation in human CC tissue. Materials and Methods: Immunohistochemical ABC and double staining studies were performed using antibodies against F1+2 and VEGF:VEGFR-2 in 59 specimens obtained from CC patients. Results: Medium and high expression of both F1+2 and VEGF:VEGFR-2 in association with CC cells and endothelial cells was demonstrated. Moreover, coexpression of F1+2 and VEGF:VEGFR-2 was observed in the cells. Conclusion: The results may suggest a possible functional interaction between thrombin and VEGF-R2 stimulation in human CC in vivo.

The course of colon cancer (CC) is frequently associated with subclinical activation of intravascular blood coagulation or clinically overt thromboembolic complications (TE) (1-3). Activation of blood coagulation proceeds not only intravascularly, but also extravascularly, at the tumor site (4-8). It is well documented that coagulation factors play a role in tumor growth and metastases formation (5, 6, 9, 10). Angiogenesis is one of the essential steps during tumor progression (11, 12). The main angiogenesis-stimulating factor is vascular endothelial growth factor (VEGF) (11, 12). Administration of antibody directed to VEGF, thus inhibiting its activity, was documented to increase overall survival of advanced CC patients and is widely used in the clinic (13). VEGF exerts its biological effect mainly through binding with receptors: FLT-1/VEGFR-1 and KDR/VEGFR-2 (14). Among various functions, tumor-induced VEGF activity leads to abnormally increased vessel permeability (11, 12). As a consequence, tumor stroma is abundantly enriched with fibrinogen and prothrombin (5-7). The activation of the blood coagulation cascade at the CC site and subsequent thrombin generation was demonstrated (15). Thrombin, apart from its main role in blood coagulation, exhibits multiple relevant biological effects, including: an increase in tumor cell adhesiveness and metastatic potential, induction of tumor cell-induced platelet aggregation (TCIPA), pro-migratory effects, mitogenesis of cancer cells (5-7, 9, 10). Interestingly, thrombin may promote angiogenesis in vivo via a mechanism independent on fibrin formation (16-18). A byproduct in the reaction of thrombin formation is prothrombin fragment F1+2 (F1+2), which presence was documented to be an indicator of activation of blood coagulation (and thrombin presence) at the various tumor sites (8). Despite the fact that thrombin was documented to exert proangiogenic activities under experimental conditions (16-18), data concerning its role in the process of new vessel formation in vivo in human cancers (e.g. CC) is obscure.

The purpose of the study was to assess the expression of F1+2, indicating the presence and localization of generated thrombin, in relation to the expression of VEGF exclusively.
when it is bound to its receptor, VEGFR-2 (indicating the presence of VEGF and VEGFR-2, as well as activation of the latter) at human CC site.

**Materials and Methods**

Colon cancer tissues were obtained at surgical resection during treatment of previously untreated 59 patients (30 women and 29 men, at the age range of 36-61 years, performance status: PS 0-1 according to WHO criteria) (19). Pathologic examination revealed G2 (G - histopathological grade of malignancy) adenocarcinoma of the colon at T2N0 clinical stage of the disease in a predominant number of the CC patients (56 cases), whereas tumor in the remaining patients (3 cases) were T3N0.

Antigens were detected with avidin–biotin complex technique (ABC) using reagents (Vectastain Kits, Vector Laboratories, Burlingame, CA, USA) which were described previously (8). A polyclonal antibody against F1+2 (8) and a specific monoclonal antibody (GV39M) binding to VEGF only when it is associated with VEGFR (VEGF:VEGFR-2) (20, 21) were utilized in the immunohistochemistry (IHC) procedures. A semiquantitative analysis of the examined protein expression was performed according to the Remmele and Stegner scale with our own modification, which was described in detail elsewhere (22). Potential co-expression of F1+2 and VEGF:VEGFR-2 was assessed employing IHC staining studies according to Dako EnVision™ (Dako, Carpinteria CA, USA) protocol provided by the manufacturer using commercially available Dako Envision™ Kit (Dako, Carpinteria, CA, USA).

Antibodies were tested on normal colon and CC tissues. Controls consisted of omission of the primary antibodies from the procedure. The results of staining of the CC tissues were compared with respective normal tissues, which were processed simultaneously. Using the ABC IHC technique both antigens were visualized as dark brown reaction products. In Dako EnVision™ technique F1+2 was visualized as red staining, whereas VEGF:VEGFR-2 gave a brown reaction product. Visual assessment of the protein expression was performed in 10 random high-power fields. The specimens were assessed by two independent, blinded observers. The study protocol was approved by the local Ethics Committee of the Medical University in Bialystok, Poland. Informed consent was obtained from the patients.

**Results**

Medium and high expression (Table I) of both F1+2 (Figure 1 A, C) and VEGF:VEGFR-2 (Figure 1 B, D) were demonstrated in association with CC cells and with endothelial cells (ECs) of tumor-supplying vessels. Moreover, coexpression of F1+2 and VEGF:VEGFR-2 was observed in both CCs, as well as in ECs of small blood vessels at the tumor site of all examined CC tissues (Figure 2). The intensity of expression of both proteins obtained in double IHC staining procedure was similar.

**Discussion**

Tumor growth creates an extra demand for oxygen, growth factors and metabolites (5, 6, 11). Formation of a new network of tumor-associated blood vessels is a prerequisite for tumor progression (6, 11, 12). The presence of VEGF was demonstrated in both cancer cells of human origin and in CC lines (23-25). Proangiogenic effect of VEGF is mainly ascribed to VEGFR-2 (14). Interestingly, the current study revealed the expression not only VEGF but also VEGFR-2 in association with CC cells. Primarily, VEGF was thought to be an angiogenesis-specific protein (11). However, subsequent experimental studies revealed that VEGFR is present not exclusively on ECs, but also on many normal cell types (e.g. macrophages, neurons, etc) as well cancer cells (e.g. VEGFR-1 on CC cells) (23, 26). IHC studies performed previously on sections derived from experimental models of cancer demonstrated that GV39M predominantly binds VEGF:VEGFR-2 localized on ECs, while to a lesser extent with cancer cells (20, 21). The study, however, demonstrated similar VEGF:VEGFR-2 staining intensity both in CC cells and ECs, indicating differences between experimental models and human-derived CC tissues. Demonstration of VEGF:VEGFR-2 on CC cells indicates the presence of an active VEGFR-2, thus suggesting its role in CC biology (independently of its role on ECs). In this regard, it is of interest that VEGF exhibits prosurvival and mitogenic activities (12, 14), which may contribute to CC growth. This, however, needs to be proved in subsequent functional studies.

In CC, activation of blood coagulation was shown to proceed not only in the vascular bed, but also in the extravascular compartment at the tumor site (8, 15). The study demonstrated the presence of F1+2 in association with CC cells and ECs of blood vessels supplying the tumor, which indicates the generation of thrombin at the sites mentioned above. Thrombin, the main enzyme of blood coagulation, contributes to multiple processes involved in cancer progression, among others, in the essential steps of angiogenesis (6, 16-18). To date, the mechanism of thrombin regulation of angiogenesis is not fully understood, and existing data are obtained exclusively from experimental studies (7, 8, 16). The most widespread thrombin receptor is PAR-1 (protease activated receptor-1, primarily designated as a ‘tethered ligand’ thrombin receptor – TLTR) (7), the presence of which was detected on ECs and cancer.
cells (7, 16, 18, 27), including CC cell lines (28). Of interest, thrombin contributes to an increased permeability of capillary vessels (11). Interestingly, recent data demonstrate that thrombin induces VEGF-induced EC mitogenesis in cultured endothelial cells (HUVEC), and this is accompanied by up-regulation of mRNA for both VEGF receptors: FLT-1/VEGFR-1 and FLK-1/KDR/VEGFR-2 (16-18). An indirect (via releasing epidermal growth factor) thrombin EC mitogenic

Figure 1. Specific staining by the ABC peroxidase technique using a polyclonal antibody against prothrombin fragment F1+2 (A), a monoclonal antibody against vascular endothelial growth factor (VEGF) only when associated with its receptor VEGFR-2 (B). Solid arrows show staining (brown reaction product) of tumor cell bodies in G2 colon cancer primary tumor, dotted arrows indicate endothelial cells of small blood vessels supplying the tumor. No staining for prothrombin fragment F1+2 (C) or VEGF/VEGFR-2 (D) was observed in normal colon tissue. Hematoxylin counterstain; original magnification: ×200 (A, B, D), ×400 (C).

Figure 2. (A) Specific staining Dako EnVision™ technique using a polyclonal antibody against prothrombin fragment F1+2, indicating thrombin generation (red staining) and a monoclonal antibody against VEGF exclusively when bound with its receptor VEGFR-2, indicating VEGF and VEGFR-2 presence and receptor activation (brown reaction product). The two colors are overlapping (solid arrows) indicating coexpression of both proteins in cancer cells (solid arrows) and endothelial cells (dotted arrow). No staining for prothrombin fragment F1+2 or VEGF/VEGFR-2 was observed in normal colon tissue (B). Hematoxylin counterstain; original magnification: ×200.
activity was also demonstrated (18). Furthermore, experimental studies revealed that thrombin increases the availability of the VEGF ligand, specifically by up-regulating the expression of VEGF by tumor cells (e.g. prostate cancer, neuroblastoma, glioma) (29-31), and by release of VEGF from platelets (32). Of interest are the present findings indicating coexpression of both F1+2 (indicating generation of thrombin) along with VEGFR-2 associated with its ligand, VEGF (indicating the activation of the receptor) in CC cells and ECs. This may suggest the role of thrombin in regulating expression of both VEGF:VEGFR-2 and VEGF in human CC. Both thrombin (through interaction with integrins) (18) and VEGF (via VEGF-dependent pathway) (23) exert antia apoptotic activity. Since the pathomechanisms of the activity are different, the effect may be additive. Furthermore, the presence of VEGF:VEGFR-2 along with F1+2 in association with CC cells may suggest existence of an autocrine loop, by which thrombin regulates cancer growth. If this is the case, introducing agents which interfere with thrombin activity as adjunctive treatment of CC patients might result in improvement of treatment results. Of interest, argatroban, an inhibitor of thrombin, was documented to inhibit thrombin-induced VEGF secretion by neuroblastoma cells (30).

Of note, even though thrombin is believed to be proangiogenic, its parental molecule, prothrombin, may also serve as a source of angiogenesis inhibitors, encoded by its cryptic domains (6). Human prothrombin fragments (F1+2), which are formed during thrombin generation, were also shown to inhibit bFGF-induced bovine capillary endothelial cell growth and angiogenesis in the CAM (33). Whether such activities exist in humans (both in ECs and cancer cells) remains obscure and deserves additional studies, but suggests a regulatory role of thrombin (activating and/or inhibiting) during angiogenesis.

Expression of F1+2 in CC tissue indicates activation of blood coagulation and generation of thrombin in the extravascular compartment at the site of CC. The presence of VEGF:VEGFR-2 in association with CC cells may indicate the role of VEGF in CC progression. Observed co-localization of F1+2 and VEGF in association with VEGFR-2 may indirectly suggest a functional interrelationship that exists between thrombin and VEGF and/or VEGFR-2 in human colon cancer in vivo.

References


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