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Co-localization of Protein Z, Protein Z-Dependent protease inhibitor and coagulation factor X in human colon cancer tissue: Implications for coagulation regulation on tumor cells

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ABSTRACT

Introduction: Several hemostatic system components, including factor X (FX), contribute to cancer progression. The Protein Z (PZ)/protein Z-dependent protease inhibitor (ZPI) complex directly inhibits factor Xa proteolytic activity. The aim of this study was to determine the antigenic distribution of ZPI and PZ, in relation to FX, as well as indicators of blood coagulation activation (F1+2 and fibrin) in human colon cancer tissue.
Materials & methods: Studies were performed on human colon cancer fragments. Immunohistochemical (IHC) ABC procedures and double staining method employed polyclonal antibodies against PZ, FX, F1+2 and monoclonal antibodies against ZPI and fibrin. *In-situ* hybridization (ISH) methods employed biotin-labeled 25-nucleotide single-stranded DNA probes directed to either FX, PZ or ZPI mRNAs.
Results: Expression of FX, PZ and ZPI in association with colon cancer cells was observed by IHC. Moreover, the presence of both F1+2 and fibrin in association with colon cancer cells was found, which indicates that blood coagulation activation proceeds extravascularly at the tumor site. Furthermore, expression of FX and PZ was visualized in association with endothelial cells. In turn, colon cancer-associated macrophages were characterized by FX , PZ and ZPI presence. The double staining studies revealed strong FX/PZ, FX/ZPI, as well as PZ/ZPI co-localization on colon cancer cells. ISH studies revealed the presence of FX mRNA, PZ mRNA and ZPI mRNA in colon cancer cells indicating induced synthesis of these proteins.
Conclusions: The localization of PZ/ZPI and FX in colon cancer cells indicates that PZ/ZPI may contribute to anticoagulant events at the tumor site. Strong co-localization of PZ/ZPI and FX in cancer cells, and the presence of the mRNAs encoding the proteins, suggests their role in the tumor's biology. However, the presence of F1+2 and fibrin at the colon cancer site also suggests that the regulation of FXa by the PZ/ZPI complex at this site is incomplete.

Introduction

More than 140 years ago Armand Trusseau pioneered observations on association between thrombotic episodes and cancer of the alimentary tract [1]. Thromboembolic complications are common findings in colon cancer patients [2–6]. Coagulation abnormalities in colon cancer patients may be subtle, being revealed exclusively in laboratory tests [7,8], or may manifest as clinically overt thrombosis or severe hemorrhage [6,9,10]. Thromboembolic episodes may also complicate treatment of colon cancer patients [8,11], since they are diagnosed in up to 29% patients undergoing surgery [8], or in 7% of patients being treated with chemotherapy [8]. They complicate the course of radiation therapy in rectal cancer patients as well [8]. Pathomechanisms of blood coagulation activation in malignancy are not fully understood yet. They may be described by so called "Virchov's

Abbreviations: ABC, avidin-biotin complex technique; T2G1, antibody directed to fibrin II (des fibrinopeptide B fibrin); AT, antithrombin; CP, cancer procoagulant; EC(s), endothelial cell(s); EPR-1, effector cell protease receptor-1; FX, coagulation factor X; FXa, activated form of coagulation factor X; F1+2, prothrombin fragment F1+2; G, grade of pathologic malignancy; HIS, in situ hybridization; HLA-DR, a class II antigen of major histocompatibility complex; IHC, immunohistochemistry; IgM, immunoglobulin M; MHC, main human compatibility; IRS, immunoreactive score; mRNA, messenger ribonucleic acid; N, number; PAR-1,-2, protease activated receptor-1,-2; PAA/PCA, platelet aggregating activity/procoagulant activity; PC, protein C; PS, protein S; PZ, protein Z; TAMs, tumor associated macrophages; TF, tissue factor; TFPI, tissue factor pathway inhibitor; TNM, tumor/nodes/metastases, classification of the clinical stage cancer; VEGF, vascular endothelial growth factor; ZPI, protein Z-dependent protease inhibitor.

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triad", which states that blood flow disturbances, damage of the vessel wall and inappropriate blood content contribute to the above mentioned complications [12,13]. Cancer cell-associated factors initiate platelets adhesion and aggregation, lead to thrombin formation, ADP generation and induce arachidonic acid metabolism [14]. The main role in thrombin generation is ascribed to cancer-dervived procoagulants [15–17]. Tissue factor (TF), cancer procoagulant (CP), procoagulant activity and platelet-aggregating activity (PCA/PAA), HLA-DR antigen of MHC (main human compatibility) class, and sialic acid residues of mucus glycoproteins synthesized by cancer cells contribute to multifactorial and cancer-specific activation of factor X (FX), which support an important role of this stage of coagulation activation in hemostatic system alterations during malignant tumor progression [2,3,13–17]. Under normal conditions activated factor X (FXa) catalyzes the reaction of prothrombin to thrombin transformation, with prothrombin fragment F1+2 (F1+2) generation as a by-product of the reaction [17]. Thrombin, being the key enzyme of coagulation, is responsible for the formation of the final product of coagulation - fibrin [17].

It was widely documented that the hemostatic system components, apart from their established role in haemostasis, contribute to cancer progression [3,13,18,19]. For example, TF (main procoagulant) is overexpressed in adenocarcinoma of the colon [20,21]. It contributes to stimulation of angiogenesis via mechanisms that are either dependent- or independent of coagulation activation, e.g. through TF signaling function resulting in the changes of expression of pro-angiogenic molecules, including VEGF [18]. Furthermore, TF facilitates distant metastases formation [18,19]. Procoagulant activity of TF leads to activation of FX, thrombin generation, platelet activation and fibrin formation, all of which influence malignant properties of the tumor [3,13]. Briefly, FXa activates nitrogen oxide synthase, induces cytokine synthesis in effector cells, promotes adhesion molecule expression and growth factors release from ECs, etc [22–24]. Experimental studies revealed that FXa, similarly to thrombin, exerts its biologic effects through activation of PAR-1 and PAR-2, and via effector cell protease receptor-1 (EPR-1), which was also observed on cancer cells [25–28]. In experimental models, FXa promotes cancer cell migration, inhibits apoptosis of the cells and contributes to inhibition of metastases formation [29–31].

In turn, thrombin facilitates distant metastases formation via an indirect influence on tumor cell-induced platelet activation and increasing adhesive properties of cancer cells and ECs [32–36]. Thrombin is a mitogenic factor for cancer cells, stimulates cancer cell secretion, retraction and migration [9,13]. Furthermore, thrombin stimulates angiogenesis, an important step in tumor growth, among others via inducing synthesis of main proangiogenic factor - vascular endothelial growth factor (VEGF) [36,37]. Increased permeability resulting from VEGF activity [37,38] allows for plasma macromolecules (among others – fibrinogen) to cross through blood vessel wall [38]. Fibrin was demonstrated to serve as a mechanistic scaffold for growing tumor and new blood vessels and may act as a protecting barrier against components of host immunologic system [13]. Furthermore, fibrin may be a reservoir of growth factors [9,13,39].

Factor Xa activity, and consequently the rate of thrombin generation, are under precise control by several regulatory mechanisms, which involve tissue factor pathway inhibitor (TFPI), antithrombin (AT) and the protein C (PC) system [17]. Recently, the presence of TFPI mRNA and protein was demonstrated in the majority (39/66 cases examined) of colon cancer tissue specimens with no presence of the protein in normal colon cells [39]. TFPI inhibits the activity of both coagulation factor Xa and tissue factor/factor VIIa (TF/VIIa) complex [17]. It contributes to the inhibition of metastases formation, particularly at an early stage of metastatic process [19]. Protein C (PC) system represents another important coagulation inhibitory mechanism [17]. In cancer patients an inverse correlation between plasma PC concentration and the risk of thromboembolic complications in post-operative period was demonstrated [40]. Furthermore, heterogeneous expression of protein C, protein S and thrombomodulin in human colorectal cancer was observed, suggesting inadequate coagulation inhibition at the tumor site, as well as pointing to the potential biological activity of the inhibitory system in modulating tumor growth [41]. Of note, activated PC (APC) was documented to promote breast cancer cell migration through its interactions with endothelial protein C recepotor (EPCR) and protease activated receptor (PAR-1) [42]. APC directly stimulates transformation of latent progelatinase A into its active form – gelatinase A (MMP-2) [43]. The enzyme plays an essential role in the proteolytic processes during angiogenesis [43], local tumor invasion and metastases formation in colon cancer [44,45]. In turn, AT inhibits thrombin proteolytic activity via thrombin – antithrombin complex (TAT) formation [45,46]. Dominant weak expression or lack of AT in colon cancer tissue was also reported [47] and decreased activity of AT was demonstrated in plasma of colon cancer patients compared to non-cancer individuals [11,48].

A mechanism for the direct inhibition of factor Xa, based on the activity of the protein Z (PZ)/protein Z-dependent protease inhibitor (ZPI) system, was recently discovered [49–52]. PZ itself does not exert any enzymatic function but serves as a co-factor in the reaction of FXa inhibition *via* ZPI [50,51,53,54]. Protein Z increases the reaction rate by more than 1000-fold, and thus efficiently contributes to diminished thrombin generation, and consequently fibrin formation [50]. The major effect of ZPI and PZ is to limit the coagulation response prior to the formation of the prothrombinase complex. Protein Z circulates in plasma in complex with ZPI. In the presence of membrane phospholipids, PZ interacts with FXa, which optimizes the inhibition of membrane-associated FXa by ZPI [55–57]. ZPI can be also activated by glycosaminoglycans on the endothelial cell surface and exerts inhibitory activity towards FXa that escapes from procoagulant phospholipids [58].

In colon cancer tissue the phenomenon proceeds not only intravascularly, but also at the tumor tissue [3,5,18,19,39,41,59]. The presence of factor X in association with colon cancer cells was reported earlier [39]. Efficient inhibition of FXa *via* the PZ/ZPI system requires the presence of the inhibitory proteins and their precise co-localization at the tumor site. The data concerning PZ/ZPI inhibition of FX in colon cancer tissue remains obscure. The purpose of the study was to determine whether FX and its inhibitory system components - PZ, and ZPI (both protein and mRNA) - are present in human colon cancer tissue. Additionally, an attempt was made to demonstrate whether the inhibitory system is efficient, namely an assessment of the well-known indicators of blood coagulation activation - F1+2 (a by-product in the reaction of thrombin generation, which is catalyzed by factor X) and fibrin (a final product of blood coagulation – documenting accomplished coagulation process) in colon cancer tissue – was performed.

Material and methods

Tissue fragments were obtained at surgical treatment of 55 previously untreated colon cancer patients (at clinical stage T2-3, N0,M0) and fixed in a buffered 4% formalin. Such buffered formalin preserves four-fold protein structure, which is necessary for antigen recognizing by an antibody. Both immunohistochemical (IHC) and *in situ* hybridization (ISH) studies were performed on adenocarcinomas of the colon (G2 – 49 cases and G3 – 6 cases) and control fragments of respective normal tissues, which were derived from the neoplasm-free surgical margins. IHC and ISH procedures are qualitative methods, which enable the assessment of the staining for examined proteins (ICH) or mRNAs (ISH) in all tissue compartments (eg. cancer cells, endothelial cells).

Staining procedures and controls for the avidin – biotin complex technique (ABC) using reagents (Vectastain Kits, Vector Laboratories, Burlingame, CA, USA) were reported in details elsewhere [60]. A semiquantitative analysis of the protein's expression exclusively in cancer cells was carried out according to the Remmele and Stegner