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Co-localization of Coagulation Factor X and its Inhibitory System, PZ/ZPI, in Human Endometrial Cancer Tissue

EWA SIERKO[1,2], EWA ZABROCKA[3], KRYSTYNA OSTROWSKA-CICHOCKA[4], PIOTR TOKAJUK[1,4], LECH ZIMNOCH[5] and MAREK Z. WOJTUKIEWICZ[1,4]

[1]*Department of Oncology, Medical University, Bialystok, Poland;*
[2]*Department of Radiotherapy, Comprehensive Cancer Center, Bialystok, Poland;*
[3]*Department of Medicine, Stony Brook University, Stony Brook, NY, U.S.A.;*
[4]*Department of Clinical Oncology, Comprehensive Cancer Center, Bialystok, Poland;*
[5]*Department of Clinical Pathomorphology, Medical University, Bialystok, Poland*

Abstract. *Background/Aim: Hemostatic system components contribute to cancer progression independently from their roles in hemostasis. It has been shown that protein Z (PZ)/protein Z-dependent protease inhibitor (ZPI) inhibit coagulation factor X (FX). The aim of the study was to analyze the expression of PZ/ZPI in relation to the main coagulation factor - FX in human endometrial cancer tissue. Materials and Methods: Immunohistochemical analysis was performed on 21 endometrial cancer specimens employing antibodies against ZPI, PZ and FX. Results: Endometrial cancer cells showed a strong expression of ZPI and PZ and medium expression of FX. Normal endometrial tissue showed no expression of ZPI, PZ or FX. Conclusion: Strong expression of PZ and ZPI in endometrial cancer cells suggests a role of these proteins in endometrial cancer.*

The estimated global incidence of endometrial cancer is approximately 382,000 per year, and nearly 90,000 of women suffering from the disease die yearly, worldwide (1). Gynecological cancer is frequently associated with thromboembolic episodes (TE) (2). The complications (*e.g.* deep vein thromboembolism, portal vein thrombosis) may precede the diagnosis of the malignant disease and accompany the treatment (3, 4). Silent or subclinical venous thromboembolic complications (VTE) occur before treatment in approximately 10% of patients with endometrial cancer (5). Thromboembolic episodes may adversely complicate surgery, external beam radiotherapy, high-dose-rate brachytherapy, chemotherapy or hormonotherapy in gynecological cancer patients (6-10). In advanced stage of endometrial cancer disseminated intravascular coagulation has been reported as well (11). It has been reported that there are changes in coagulation factors prior to any treatment for endometrial cancer, suggesting that the disease may result in a procoagulant state (12, 13). Namely, increased levels of fibrinogen, thrombin–antithrombin complex (TAT), and prothrombin fragment F1+2 have been observed compared to non-cancer individuals (12, 13). One of the important steps of coagulation activation in cancer patients has been ascribed to the activation of factor X (FX) (14). Many cancer-specific stimuli have been recognized to trigger its activation, *e.g.* tissue factor (TF), cancer procoagulant (CP), procoagulant activity and platelet-aggregating activity (PCA/PAA), HLA-DR antigen of MHC (main human compatibility) class, as well as sialic acid residues of mucus glycoproteins, which are synthesized by cancer cells (14, 15). Numerous studies strongly suggest that the hemostatic system components contribute to cancer progression independently from their established roles in hemostasis (14, 16). Factor Xa stimulates cytokine synthesis in effector cells, activates nitrogen oxide synthase, induces adhesion molecule expression as well as the release of growth factors from endothelial cells (ECs) (17, 18). It can also activate endothelial protein C receptor (EPCR) and protease activated receptor-1, which are known to play a role in cancer growth and dissemination (19). FXa, similarly to thrombin, exerts its biologic effects *via* activation of protease activated receptor-1 and -2 (PAR-1 and PAR-2) (20-22). The presence of PAR-1 in endometrial cancer tissue has been confined to the highly aggressive, high grade endometrial carcinoma, but not in benign tumors of the endometrium (20). PAR-2 expression, both at the mRNA and protein level, has been

Correspondence to: Ewa Sierko, MD, Department of Oncology, Medical University, 12 Ogrodowa St., 15-027 Bialystok, Poland. Tel: +48 856646786, Fax: +48 856646783, e-mail: ewa.sierko@iq.pl

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shown to be significantly increased in uterine endometrial cancers, and to correlate with clinical stage, cancer dedifferentiation as well as with myometrial invasion in comparison to normal endometria (23). Significant correlations between PAR-2 histoscores and mRNA levels with microvessel density in uterine endometrial cancers have been observed suggesting that PAR-2 contributes to endometrial cancer progression *via* its angiogenic activity (23). In experimental models, FXa has been shown to facilitate cancer cell migration, inhibit apoptosis and contribute to inhibition of metastases (24, 25). Thrombin is known to facilitate distant metastases by influencing tumor cell-induced platelet activation, as well as by increasing the adhesive potential of cancer cells and ECs (26-29). Thrombin has also been shown to stimulate cancer cell detachment and migration (14, 30) and to facilitate angiogenesis via, among others, inducing synthesis of proangiogenic factor - vascular endothelial growth factor (VEGF) (27, 28). VEGF is one of the factors contributing to the aggressive phenotype of endometrial cancer and the pronounced vascular invasion (31). VEGF activity results also in increased permeability of the endothelium (32, 33), which facilitates the entry of plasma macromolecules (*e.g.* fibrinogen) to the extravascular space (33). In turn, fibrin serves as a mechanistic scaffold for proliferating cancer cells and newly formed vessels, a protecting barrier against components of the host's immune system (14), and a reservoir of growth factors (14, 30, 32).

Recently, it was shown that factor Xa is directly inhibited by the activity of the protein Z (PZ)/protein Z-dependent protease inhibitor (ZPI) system (34). PZ serves as a co-factor in the reaction of FXa inhibition *via* ZPI, but has no enzymatic function itself (35-37). It has been shown that PZ increases the reaction rate by more than 1,000-fold, efficiently facilitating inhibition of thrombin generation (35). The major role of the ZPI/PZ system is to inhibit the coagulation response prior to the formation of the prothrombinase complex. Protein Z and ZPI circulate in plasma as a complex. In the presence of membrane phospholipids, PZ interacts with FXa. This facilitates the inhibition of membrane-associated FXa by ZPI (38). ZPI can also be activated by endothelial cell surface glycosaminoglycans and inhibit FXa, which escapes from procoagulant phospholipids (39).

The aim of the study was to analyze the solid phase interaction between PZ/ZPI and the main coagulation factor - FX in human endometrial cancer tissue.

Materials and Methods

Tissue fragments were obtained at surgical treatment of 21 previously untreated endometrial cancer patients (at clinical stage FIGO II) and fixed in 4% buffered formalin. Immunohistochemical (IHC) studies were performed on adenocarcinomas of the endometrium (G1 – 6 cases, G2 – 12 cases and G3 – 3 case) and control fragments of respective normal tissues, which were derived from the neoplasm-free surgical margins.

Staining procedures and controls for the avidin – biotin complex technique (ABC) using Vectastain Kits (Vector Laboratories, Burlingame, CA, USA) have been previously described in detail (40). A semi-quantitative analysis of protein expression in cancer cells was carried out according to the Remmele and Stegner scale with our own modification (reported elsewhere) (41, 42). The analysis accounted for the diversity of staining observed in our studies (42, 43). Both percentage of positively stained cancer cells (A) as well as the intensity of staining (B) are given in numerical values. Immunoreactive score (IRS) was a product of values A and B (IRS=A×B). Immunoreactive score (IRS) values between 1-4 were interpreted as weak, 5-8 as medium, and 9-12 as strong protein expression (42, 43). A monospecific antibody against homogeneous, plasma-derived human PZ was prepared in rabbits, and purified from immune sera by protein A-Sepharose chromatography (44). Specific monoclonal mouse anti-human ZPI IgM (4249.2) was generously provided by Dr. George J. Broze Jr. (Division of Hematology, Barnes-Jewish Hospital, St. Louis, MO, USA) (45), while polyclonal antibodies specific for human FX - by Dr. David Stump (Genentech, South San Francisco, CA, USA) (46). In the control specimens, the primary antibody was omitted from the procedure. In the ABC immunostaining procedure, antigen staining was detected as a dark brown reaction product.

Visual assessment of protein expression was performed in 10 consecutive high-power fields by two independent observers. The results of IHC studies of endometrial cancer tissues were compared with the results obtained from respective normal endometrium, which were processed simultaneously. The study protocol was approved by the local Ethics Committee of the Medical University in Bialystok, Poland (approval number R-I-002/256/2003) Informed consent was obtained from the patients.

Results

Strong expression of ZPI (IRS=9) was observed in endometrial cancer cells (Figure 1A). Similarly, endometrial cancer cell bodies were characterized by strong staining intensity of PZ (IRS=9.5) (Figure 1B). Medium expression of coagulation factor X (IRS=8-9) was revealed in association with cancer cells in endometrial cancer specimens (Figure 1C). No differences in intensity of staining for ZPI, PZ and factor X were observed in G1, G2, G3 endometrial cancer (IRS – 9-9.5). No staining for the above-mentioned proteins were observed in normal endometrial tissue (Figure 1D, E and F).

Discussion

In the cancer patients, venous thromboembolism has been found to be associated with poor prognosis (47). Thrombosis is also the second leading cause of death in this group (16). It is known that blood coagulation can occur not only intravascularly but also extravascularly, within the tumor environment (48). The products of blood coagulation activation have been reported to contribute to angiogenesis and progression at the tumor site as well as distant

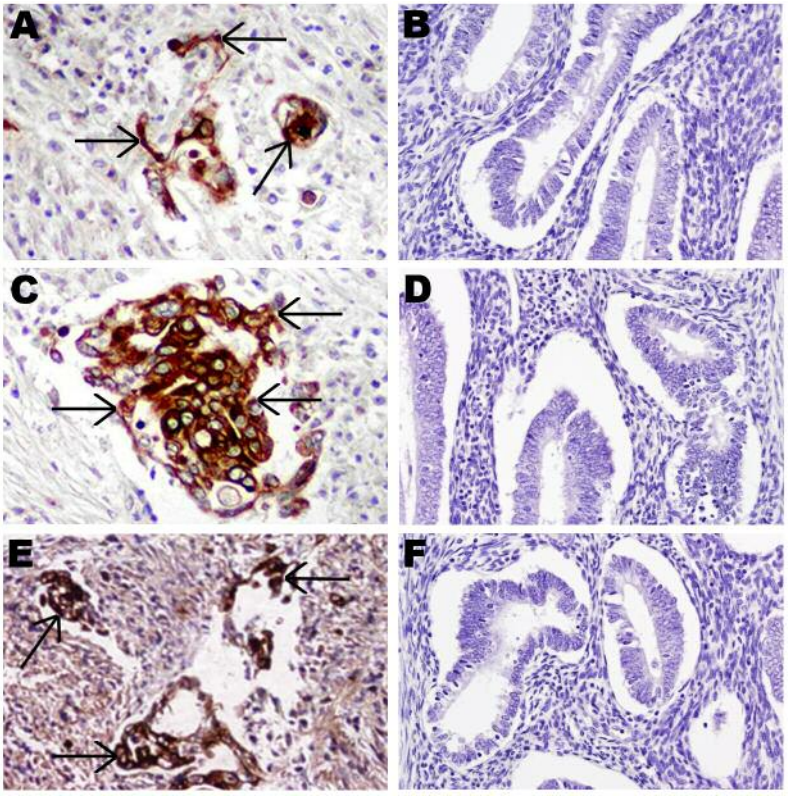

Figure 1. *Immunohistochemical staining (brown reaction product) by ABC peroxidase technique, using polyclonal antibodies against protein Z – PZ- (A), coagulation factor X -FX- (E), as well as monoclonal antibody against protein Z-dependent protease inhibitor –ZPI- (C). Arrows show staining of tumor cell bodies in endometrial cancer cells. No staining for PZ (B), ZPI (D) or FX (F) was observed in normal endometrial tissue. Hematoxylin counterstain; original magnification ×100 (Fig. A, B, D, E, F) and ×200 (Figure C).*

metastases (27, 28, 48). However, the exact mechanism of extravascular blood coagulation at tumor sites has not been fully understood. Uccella *et al*. have reported on the ability of endometrial cancer tissue to synthesize fibrinogen (49) – a protein which may increase metastatic capacity of tumor cells (50). Endometrial cancer cells have also been found to express tissue factor after induction by epidermal growth factor, resulting in increased invasive potential (50).

Factor X plays an important role in blood coagulation activation pathway (14). Additionally, it is known to be involved in various biological processes within the tumor environment (48). The present study showed that the expression of factor X was associated with endometrial cancer cells but not with normal endometrial tissue. The results of previous studies showing the presence of factor X mRNA in cancer tissue indicate that this coagulation factor can be synthesized by malignant cells (46). Factor X expression has also been observed in gastric and colon cancer cell bodies (46).

Physiologically, increased expression of coagulation factors leads to an increase in the expression of their inhibitors. Factor X activity is regulated by inhibitory mechanisms, which include tissue factor pathway inhibitor (TFPI), antithrombin, and the PZ/ZPI system (34). However, the data on the PZ/ZPI in endometrial cancer site are obscure. The present study revealed PZ and ZPI expression in endometrial cancer cells, similarly to previous studies that have demonstrated PZ/ZPI presence in breast, colon, gastric and non-small cell lung cancer tissue (44-46, 51-54). These studies have also revealed the presence of PZ/ZPI mRNA at cancer sites, implicating *in loco* synthesis of these proteins. Additionally, ZPI overexpression has been identified in pancreatic endocrine tumors and their metastases to the liver (55). The presence of both PZ and ZPI, along with FX, in association with endometrial cancer cells may indicate that these proteins modulate coagulation or other processes involved in endometrial cancer pathology at the tumor site.

Except for a putative role in tumor biology, the proteins involved in hemostasis may have the potential to be tumor markers. Gynecological cancer patients have been reported to have significantly higher plasma levels of prothrombin fragment F1+2 and thrombin-antithrombin complex compared to healthy women (56). Although the synthesis of

PZ/ZPI by cancer cells has been reported, it is still unclear if this ectopic expression affects plasma ZP/PZI levels. This may be difficult to investigate in the context of recent findings suggesting that ZPI (but not PZ) may be an acute phase reactant (57). This is reflected in the study by Yoshida *et al*., which revealed a significant increase in median ZPI levels together with CRP after tumor-related gynecological surgery (58). A significant correlation between ZPI and CRP or fibrinogen has also been found in colorectal and pancreatic cancer patients (59). The same study showed no correlation between PZ levels and CRP or fibrinogen, which may indicate that high levels of PZ are rather unrelated to the inflammatory state and can be attributed to the ectopic synthesis of PZ by cancer cells. On the contrary, another study has shown lower PZ levels in cancer patients compared to healthy individuals (60). However, all tumors in this study were analyzed collectively, therefore the analysis of PZ levels in individual tumor types is encouraged. Recently, PZ role as a marker of early detection of ovarian cancer has been suggested (61).

Strong expression of PZ and ZPI in association with endometrial cancer cells revealed by the present study suggests a putative role of these proteins in endometrial cancer biology. The intensity of ZP/PZI expression appears not to be influenced by tumor grade. Further studies on PZ/ZPI role in cancer pathology are needed to fully investigate the role of this system in cancer.

Conflicts of Interest

The Authors declare no conflicts of interest regarding this study.

Authors' Contributions

Ewa Sierko – concept of the study, supervision, collecting material, performing experiments, interpreting results, writing the manuscript, approval of the text of the manuscript. Ewa Zabrocka – interpreting the results, writing the manuscript, approval of the manuscript; Krystyna Ostrowska- Cichocka – collecting material, performing experiments, interpreting the results, approval of the manuscript; Piotr Tokajuk - performing experiments, interpreting the results, literature searching, approval of the manuscript; Lech Zimnoch – interpreting the results, approval of the manuscript; Marek Z. Wojtukiewicz – concept of the study, supervision, approval of the manuscript.

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