

Endothelial cell activation and proliferation in ovarian tumors: Two distinct steps as potential markers for antiangiogenic therapy response

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Abstract. Ovarian cancer is the second leading cause of cancer-related death in women worldwide. Since most patients are diagnosed in advanced disease stages, the starting point, early steps and molecular mechanisms of ovarian cancer angiogenesis are still incompletely characterized. Most immunohistochemical studies for assessment of microvessel density (MVD) in ovarian tumors are based on CD31, CD34 and CD105 immunostaining of tumor blood vessels. Yet, the proliferative status of tumor blood vessel endothelial cells has not as yet been used in the assessment of tumor blood vessels. The present study investigated the Ki67 proliferative index of tumor blood vessel endothelial cells highlighted with the CD34 panendothelial marker and the CD105 endothelial marker in ovarian cancers by antigen co-localization with a doublestaining immunohistochemical method. Lack of co-localization of CD105 and Ki67 in all types of ovarian tumors together with the presence of CD34⁺/Ki67-positive endothelial cells suggest that endothelial cell activation and proliferation are distinct steps in ovarian tumors. Differences in the proliferation index were observed in endothelial cells from blood vessels of the tumor core and those of the tumor peripheral zones. Potential specific targeting of activated and proliferating tumor blood vessels may provide clues for improving antiangiogenic therapy efficiency.

Introduction

Previous classification of tumor blood vessels as immature, intermediate and mature (1), as well as microvessel density (MVD) were certified as prognostic factors in ovarian

carcinomas (2,3). Tumor angiogenesis during ovarian cancer development involves both sprouting and intussusception mechanisms (4) as was demonstrated for most human tumors in several experimental models (5-7). There already exist well-known signaling pathways that induce the formation of new blood vessels; yet, somewhat surprisingly, the targets of these signals, endothelial cells are less characterized (8). Thus, which endothelial cells initiate the processes involved in budding, migration and proliferation and how they are activated are yet unknown.

Although there is no specific marker for tumor vasculature, several potential candidates with therapeutic implications have been identified. Due to these inconclusive findings, several researchers have attempted to identify a marker with high specificity for activated endothelial cells. Among them, one of the most promising is endoglin, preferentially expressed in angiogenic endothelial cells. This property is the main reason for the identification of CD105 as a primary target for tumor imaging, prognosis and therapy (9,10).

Endoglin is expressed as a 180-kDa cell surface transmembrane homodimeric protein. It has an external domain which recognizes TGF- β 1, and the encoding gene is located on chromosome 9q34 (11). Although the mechanisms and functions of CD105 in angiogenesis are not fully characterized, it was reported that, in CD105-knockout mice, multiple cardiovascular abnormalities occur leading to death at an early embryonic stage (12). Lack of CD105 induces abnormal steps in blood vessel maturation and remodeling processes, leading to dilation and blood vessel rupture (13).

CD105 has high specificity for tumor vasculature when compared with panendothelial markers (14). MVD assessed using CD105-stained specimens was correlated with survival in breast cancer, unlike CD31-staining methods (15). Similarly, in prostate cancer, CD105 expression was correlated with tumor stage, Gleason score, metastasis, proliferation index and survival (16). These correlations were not found in specimens stained for von Willebrand Factor.

Based on these findings, CD105 is an ideal target for therapy as specific antibodies do not react with normal endothelial cells.

On the other hand, although MVD assessed by any panendothelial marker has prognostic value, it does not represent

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valuable criteria for response to antiangiogenic therapy (17). The endothelial cell proliferation index as assessed by Ki67, may represent a predictive factor for the response to antiangiogenic therapy.

Ki67 is a cell proliferation marker (18), which defines both tumor and endothelial cell proliferation. Ki-67 can be found in all active cell cycle steps (G_1 , S, G_2), while it is not expressed in dormant cells (G_0 phase). Several studies have associated endoglin expression with a high endothelial cell proliferation rate *in vitro* (19), yet few studies regarding co-expression of CD105 and Ki67 in endothelial cells from tumor blood vessels are currently available (20,21). The study of CD105/Ki67 expression in tumor blood vessels from ovarian carcinomas has not been previously carried out.

For these reasons, we proposed to investigate Ki67 and endoglin expression compared with CD34/Ki67 expression by double immunostaining applied on selected specimens of ovarian tumors to assess the sequence of endothelial cell activation and proliferation events in the tumor and peritumor area.

Materials and methods

Patient selection. Sixty-two female patients diagnosed with ovarian tumors were retrospectively selected during a four-year period. All patients had complete clinicopathologic and postsurgical evaluation data. Patients with ovarian tumors were well characterized in regards to invasion (both, local and distant), and surgical protocols applied for each patient. A signed consent from each patient included in this study was obtained.

All procedures were carried out according to the principles embodied in the Declaration of Helsinki and were approved by the Institutional Review Board of 'Victor Babes' University of Medicine and Pharmacy, Timisoara, Romania.

Specimens and description of the histopathologic primary processing methods. Tumor specimens were surgically removed and carefully selected by choosing the most representative parts which included both tumor tissue and adjacent normal ovarian tissue. Tumor parts with necrosis and extensive hemorrhages were avoided. Small tumor tissue biopsies (10x10x3 mm) were washed in saline solution, fixed in 10% buffered formalin for 24 h and paraffin embedded. From each paraffin-embedded specimen, we constructed 5- μ m serial sections mounted on silanized slides. One slide from each case was stained with routine hematoxylin and eosin (H&E) method for histopathologic evaluation and also for case selection for immunohistochemical procedures.

Immunohistochemistry. Activation and proliferation of tumor endothelial cells were assessed by using anti-CD105 antibodies and immunohistochemistry for activated tumor blood vessels. Double immunolabeling of tumor blood vessel endothelial cells with the anti-CD105 or anti-CD34 antibody and the anti-Ki67 antibody was applied to detect the proliferative status of the endothelial cells in the ovarian tumors. Incubation with primary antibodies was followed by the use of labelled streptavidin biotin method (Advance/HRP, Dako, Glostrup, Denmark, for CD105 immunostaining) and EnVision G Doublestain detection system (Dako, for double

immunostaining). 3,3'-Diaminobenzidine was used as a chromogen for the visualization of CD105-positive blood vessels. The same chromogen highlighted Ki67-positive nuclei in the doublestaining detection method together with fast red chromogen used for CD34-positive endothelial cell visualization. All immunohistochemical procedures were performed with a fully automated workflow by using PT Link (for dewax and antigen retrieval procedures) and Dako Autostainer immunostaining system.

Microscopic evaluation and statistical analysis. Microscopic evaluation was carried out using a Nikon Eclipse E600 microscope. Image acquisition was performed with a Coolpix 950 digital camera followed by image analysis using Lucia G software, version 13. Assessment of doublestaining was carried out only for the presence of both CD105-/Ki67-positive or CD34-/Ki67-positive signals to avoid the quantification of tumor cells which were also positive for Ki67 proliferation. Particular morphological features, MVD and proliferative status of ovarian tumor blood vessels were the main data analyzed in the present study.

Results

Upon microscopic evaluation of the H&E-stained tumor specimens, we found four main histopathological types of ovarian tumors: serous carcinomas (62%), mucinous carcinoma (18%), clear cell carcinomas (6%) and ovarian germ cells tumors (8%). Most of the ovarian tumors described above had a G_2 tumor grade (58%), followed by G_3 (39%) and G_1 (3%).

Most of the CD105-positive tumor blood vessels were observed inside the tumor area and also CD105-positive vessels were found at the border between the tumor cells and adjacent stroma. All CD105-positive tumor blood vessels had small size, were irregular in shape and had split lumen (Fig. 1a). An increased number of CD105-positive tumor blood vessels was noted in the tumor areas with inflammation yet these findings were not statistically significant (Fig. 1b). Low differentiated tumors had an increased MVD as assessed for CD105-positive tumor blood vessels (Fig. 1c). Plexiform grouping of CD105-positive tumor blood vessels in G_3 ovarian tumors made, in some cases, the assessment of MVD difficult (Fig. 1d). The median value of MVD in the tumor area had an average of 23.44 vessels/field compared with the peritumor area which had a MVD value of 14.33 vessels/field (Fig. 1e and f). Double staining for CD105 and Ki67 revealed that CD105-positive vessels did not express the Ki67 proliferation marker (Fig. 2). This was a constant finding for all types of ovarian tumors which suggests that tumor endothelial cell activation and proliferation are distinct steps of vascular network development in ovarian tumors.

Evaluation of CD34/Ki67 doublestaining was carried out inside the tumor and around it for all types of ovarian tumors. There was a relationship between CD34-positive blood vessel morphology and Ki67 expression in endothelial cells lining these vessels. Most of the large vessels with regular lumen were lined by dormant endothelial cells without expression of the Ki67 proliferation marker (Fig. 3a), but few exception were found (Fig. 3b). The lack of Ki67 expression in CD34-positive endothelial cells lining the peritumor blood vessels suggest a low endothelial proliferation rate in blood vessels adjacent to

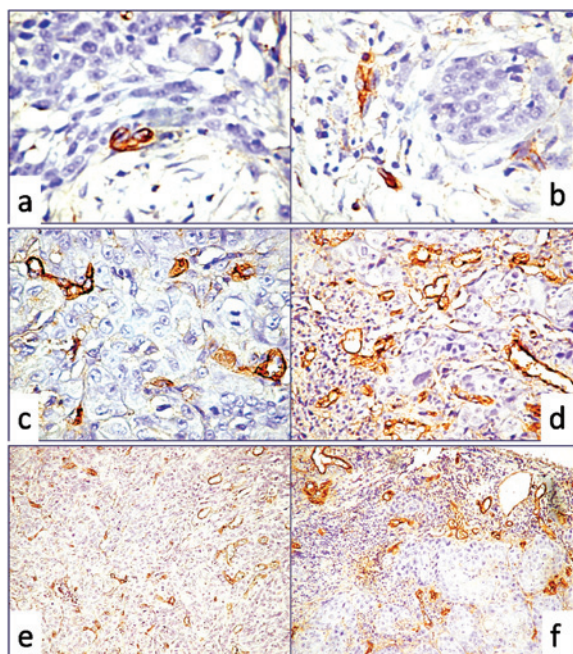


Figure 1. Endoglin (CD105) expression in ovarian tumors. Small, irregular CD105-positive tumor blood vessels (a), more numerous inside the tumor (b), especially in undifferentiated carcinoma (c), forming intratumor vascular plexiform structures (d). Differences in CD105-positive immunostaining observed between the intratumor (e) and peritumor (f) area.

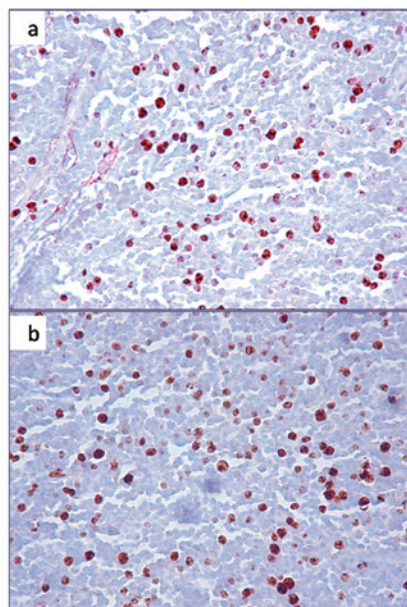


Figure 2. Intratumor view of double immunostaining for CD105/Ki67 antigens. We noted that CD105-positive (red) intratumor blood vessels were not colocalized with Ki67 nuclear staining (brown) in endothelial cells from tumor blood vessels (a). Ki67 was also positive in the nuclei of tumor cells (b).

tumor tissue (Fig. 3c-e). This was a homogeneous finding independent of the blood vessel size and morphology. Intratumor blood vessels had a small lumen and presented numerous Ki67-positive proliferative endothelial cells. Co-expression of CD34 antigen and Ki67 proliferation marker was the most frequent pattern for intratumor blood vessels, and the endothelial proliferation index was approximately 11.2% (Fig. 3f) compared with 4.5% found in the peritumor area.

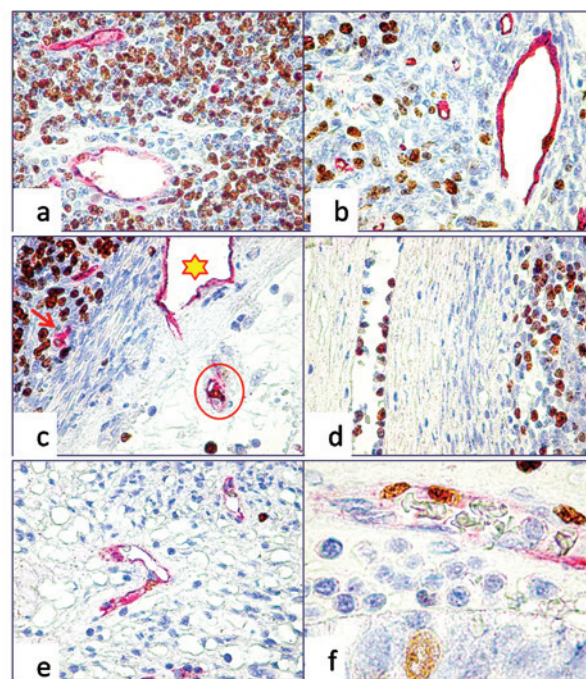


Figure 3. CD34/Ki67 doublestaining of endothelial cells from ovarian cancer tumor blood vessels. Large blood vessels lined by proliferative endothelial cells (a and b) mixed with small CD34-positive blood vessels. All three types of tumor blood vessels were noted in ovarian cancer: CD34-positive immature vessels (c, arrow), large mature blood vessels with sprouting phenomenon without endothelial proliferation (c, asterisk) and intermediate CD34-/Ki67-positive blood vessels (c, circle) with luminalization of endothelial cell cytoplasm without inside blood flow. Large blood vessels with a high rate of endothelial cell proliferation and no CD34 immunostaining (d) compared with CD34-/Ki67-positive blood vessels noted in (f). Proliferation of endothelial cells from an intratumor sprouting vessel (e).

Discussion

Epithelial ovarian cancer is the second leading cause of cancer-related death in women worldwide, and investigation of antiangiogenic agents in this disease has demonstrated antitumor activity both as a monotherapy and in combination with cytotoxic chemotherapy (22). Many questions regarding their use in treatment, their use as single agents or in combination with chemotherapy or which biomarkers predict efficacy remain unanswered (22,23).

As most patients are diagnosed at an advanced stage, they have a high morbidity and mortality rate (24). The starting point, early steps and molecular mechanisms of ovarian cancer angiogenesis are still incompletely characterized (25). Most immunohistochemical studies for assessment of MVD in ovarian tumors are based on CD31, CD34 and CD105 immunostaining of tumor blood vessels (26,27) but the proliferative status of tumor blood vessel endothelial cells has not been previously performed.

CD34/Ki67 and CD105/Ki67 doublestaining methods applied in this study revealed that CD105-positive activated endothelial cells had no positive reaction for the Ki67 proliferation marker. Moreover, our results showed a different proliferative index between the tumor blood vessel endothelium from the tumor core and its peripheral counterparts. This finding could partially explain the ineffective antiangiogenic and/or antivascular therapy and the recovery of

tumor angiogenesis in ovarian cancers as reported in several studies (26,28).

Recent studies consider the VEGF/VEGFR axis as the main target for antiangiogenic therapy in ovarian cancer (28-30).

CD34 protein is a member of a family of single-pass transmembrane sialomucin proteins that exhibit expression in early hematopoietic and vascular-associated tissue. CD34 is also an important adhesion molecule. Lack of CD34 immunostaining in some blood vessels lined by several Ki67-positive endothelial cells as we found in this study suggest that ovarian cancer tumor blood vessels are more permeable than normal blood vessels and this could be an important factor for vascular invasion and metastasis.

No data are available concerning endothelial cell proliferation and activation as distinct steps and potential distinct therapeutic targets in ovarian cancer. Our study suggests that ovarian tumor blood vessels are heterogeneous, do not have the same proliferative status or expression of markers concurrently and may respond in a different way to antiangiogenic therapy.

Based on this study, endothelial cell activation represents a distinct step in ovarian tumor-associated angiogenesis. Lack of co-localization of CD105 and Ki67 by immunohistochemistry sustains this hypothesis. CD105 highlights more specific intratumor blood vessels as we already demonstrated by differences between intratumor and peritumor MVD. Also, a high proliferation rate in CD34-positive endothelial cells inside the tumor and the lack of this proliferation in CD105-positive intratumor blood vessels suggest that activation and proliferation of endothelial cells are distinct steps as well as specific markers for tumor-associated angiogenesis in ovarian cancer. Their evaluation could be useful for monitoring antiangiogenic and/or antivascular therapy response.

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