CD105/Smooth muscle actin double immunostaining discriminate between immature and mature tumor blood vessels

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Abstract
The aim of the study was to demonstrate the value of the double immunostaining for endothelial and perivascular cell to discriminate mature from immature tumor-associated blood vessels in mammary carcinoma. We used the specific endothelial marker CD105 to highlight the activated endothelial cells and antibodies against smooth muscle cell actin (SMA) for perivascular cells, applying Envision Doublestain system (HRP/DAB–APAAP/Fast Red). We found an inverse correlation between the immunoexpression of CD105 and SMA in normal vessels and a strong positive signal for CD105 in the intratumor single endothelial cells and immature vessels. Only few blood vessels were positive for both CD105 and SMA within the tumor area. The signal for the endothelial marker was weak and inconstant, and significantly diminished when the SMA immunoexpression was increased for the same vessel. The differentiation between vessels with and without perivascular cells coverage using double immunostaining for CD105/SMA may be an important step in the selection of the mammary tumors, which could have a high grade of responsiveness to antiangiogenic therapy with monoclonal antibodies against CD105 antigen.

Keywords: angiogenesis, mammary carcinoma, CD105, Smooth muscle cell actin (SMA), tumor blood vessels.

Introduction
Angiogenesis is a fundamental process by which new blood vessels are formed from preexisting ones. Pathologic angiogenesis is a hallmark of cancer and various ischemia and inflammatory conditions. Tumor growth and metastasis are angiogenesis-dependent. Antibody targeting tumor-associated vasculature is a promising therapeutic approach in human cancer. However, a specific cell membrane marker for endothelial cells of tumor vasculature has not been discovered yet. Endoglin (EDG) was initially reported as a human leukemia-associated cell-membrane homodimer glycoprotein [1].

EDG is a proliferation-associated antigen of endothelial cells and expressed more abundantly in the vascular endothelium of tumor tissues than in their normal counterpart. In addition, EDG is an ancillary transforming growth factor beta (TGF-beta) receptor [2].

In solid tumors, CD105 is found on endothelial cells of both peri- and intratumor blood vessels and in tumor stroma components. In particular, CD105 is largely expressed in tumor vessels, as demonstrated in breast, prostate and gastric cancer; rarely, CD105 is expressed in the cytoplasm of neoplastic cells.

Among potential therapeutic strategies to induce tumor regression by blocking tumor blood supply, an intriguing approach relies on the selective targeting of cell surface molecules overexpressed by endothelial cells of tumor-associated blood vessels.

In this setting, emerging in vitro and in vivo preclinical evidence identifies CD105 as a cell membrane glycoprotein representing a prime vascular target to implement innovative antibody-based diagnostic and therapeutic strategies shared by human neoplasia of different histotype.

Double immunostaining for an endothelial and perivascular marker on the same section could select the patients that could respond properly to an antibody-based targeted therapy against tumor neovascularisation for both systemic and/or intratumor administration.

Material and methods
Because this study had mainly a technical connotation, we used five cases of breast carcinoma, three fibroadenomas and two cases of normal mammary gland as tissue control. The biopsies were collected by open surgery from breast masses clinically or mammographically detected.

The specimens were formalin fixed and paraffin embedded using standard protocols. Fifteen sections 5 µm thick were obtained from each case.

We performed Hematoxylin–Eosin staining method to establish the pathologic diagnosis and grade. For the double immunostaining we applied Envision Doublestain system (DakoCytomation).

We used anti-CD105 antibody, clone SN6h (HRP method and 3,3’-diaminobenzidine as chromogen) to highlight endothelial cells of neovessels, and anti-smooth muscle actin (ready-to-use), clone 1A4
found only mature vessels with a lot of perivascular peritumoral fat. In the stroma of fibroadenomas we linked to invasion of the neoplastic cells in the late stages.

The sections were counterstained with modified Lille’s haematoxylin for three minutes. The mounting medium was aqueous, because Fast Red is alcohol soluble. All reagents for immunohistochemistry were from DakoCytomation (Denmark).

We used Nikon Eclipse E600 microscope for examination and interpretation of the results. We noticed the number, distribution and type of intratumor positive vessels for CD105 (brown) and SMA (red).

We also studied the morphology and immunohistochemical features of peritumoral vessels.

Results

On routine haematoxylin and eosin stain, the neoplastic tissues were found in five cases of ductal invasive carcinoma with diffuse sheets, well-defined nests, and cords or as individual cells. Vascular invasion without perineural invasion was detected in three cases. The fibroadenoma had the classic microscopic feature and terminal ductal lobular units linked to intralobular and interlobular stroma were recognized in normal breast tissue. Blood vessels of the normal mammary stroma were negative for CD105 and positive (red) for SMA.

We detected activated endothelium positive for CD105 outside the tumor linked to sites reach in inflammatory infiltrate. These vessels were also variable positive for SMA (Figure 1a). The vessels that emerged from the preexistent one (positive only for SMA) became positive for CD105 too (Figure 1b).

Around the tumor there were large vessels with sinuous branched lumen lined by CD105 positive endothelium only, and vessels with small lumen and tendency to branch through the neoplastic tissue. In the latter type of vessels, we found positive reaction for both CD105 and SMA antibodies (Figure 2, a and b).

Within the tumor there were found all three types of tumor neo-vessels: single endothelial cells (immature), intermediate and mature vessels. Almost all neovessels of the neoplastic tissue have features of single endothelial cells (which predominate) and intermediate vessels and have no signal for anti-SMA antibodies (Figure 3, a and b).

Only few tumor vessels were of mature type, and expressed SMA with weak intensity and discontinuous pattern. In such vessels the signal for the CD105 stain was diminished in comparison with small vessels without reaction for SMA (Figure 3c).

We found large vessels positive only for CD105 inside the adipose tissue around the tumor, closely linked to invasion of the neoplastic cells in the peritumoral fat. In the stroma of fibroadenomas we found only mature vessels with a lot of perivascular cells around them positive for SMA and no endothelial reaction for anti-CD105 antibodies. These vessels were located into the stroma and they were arranged near the collapsed ducts.

Discussions

Angiogenesis, the formation of new blood vessels from preexisting ones, is accompanied by the synthesis of antigens on tumor endothelial cells and of novel ECM components [3]. Angiogenesis is a rare phenomenon in healthy adults, occurring only locally and transiently under distinctive physiological conditions, such as wound healing, inflammation, and the female reproductive cycle. Microvessel density (MVD) has been reported to be an independent prognostic indicator of outcome in a variety of human malignancies [4]. Pan-endothelial markers (CD31, CD34, factor VIII) stain endothelial cells but sometimes fail to localize small tumor blood vessels and therefore underestimate the MVD in tumors [5, 6].

Endoglin is essential for angiogenesis and vascular development and is strongly expressed on vascular endothelium of tumor tissues but less so on that of normal tissues [7]. CD105 (endoglin) is predominantly expressed in angiogenic endothelial cells and upregulated by hypoxia [8].

We also registered a strong reaction for CD105 in the activated endothelium (from tumor blood vessels and vessels of inflammatory sites) compared with normal mammary gland and fibroadenomas that were negative for this marker. Moreover, CD105 inhibits apoptosis in activated endothelial cells from tumor blood vessels [9].

Currently, great interest is focused on angiogenesis and its potential clinical implications in cancer, and vascular targeting represents a highly promising alternative to the direct engagement of therapeutic TAA on neoplastic cells. Certain anti-endoglin monoclonal antibodies (mAbs), termed SN6 series mAbs, inhibited angiogenesis, tumor growth and metastasis in mice in a dose-dependent manner [10].

In patients with breast cancer, shed CD105 levels were markedly elevated in plasma samples compared with healthy controls. CD105 levels were significantly increased in those patients who subsequently developed distant metastasis.

Takahashi N et al. [11] had confirmed these findings in serum samples from patients with colorectal, breast and other types of cancer. Patients with metastatic disease had significantly elevated CD105 levels in comparison with metastasis-negative individuals and healthy controls.

Li C [12] showed a correlation between MVD using CD105 antibody and elevated serum CD105 and poor prognosis in colorectal cancer. Also, it was demonstrated that CD105 – MVD is correlated with strong expression of VEGF in colon cancer and the high levels of both markers suggest a deeper invasion and lymph node metastasis in gastrointestinal cancers [13].
**Figure 1** – Preexistent vessels with red signal for SMA only (upper left) together with small blood vessels with activated endothelium positive for CD105 (center, brown), and negative for SMAct. (a). Detail from a preexistent vessel CD105-/SMA+ which acquire CD105 signal when it become branched (b).

**Figure 2** – Peritumoral small blood vessels, positive for both Cd105 and SMA (a). Note the diminished signal for SMA (red) as the vessels become branched (b).

**Figure 3** – Types of intratumoral blood vessels founded in ductal invasive carcinoma: (a) single endothelial cells (no lumen, CD105+/SMAct-); (b) immature type (with lumen, CD105+/SMAct-); (c) mature tumor blood vessels with signal for SMAct and diminished reactivity for CD105.
Moreover it was reported that there are differences of endoglin expression between polyoid cancers of the colon and non-polyoid type [14].

Targeting of CD105, as therapeutic antiangiogenic approach in cancer, has been extensively investigated in severe combined immunodeficiency mice bearing human breast tumors. The results of these studies demonstrated a long lasting suppression of tumor growth and metastasis by systemic administration of radiolabeled or immunotoxin-conjugated anti-CD105 mAb [15].

According with Gee S et al. [16] there are differences in the behavior to the therapy between pericyte-positive and pericyte-negative tumor blood vessels in human tumors. Quantitation of single endothelial cells may be useful because it provides an indicator of tumor angiogenic activity distinct from, but related to, pericyte coverage [17].

In our study, there are numerous single endothelial cells inside the tumor together with intermediate tumor blood vessels without positive red signal for SMA around them. This is in concordance with results published by Wilkstrom P et al. [18] on neoplastic prostate tissue when they applied double immunostaining for the same markers. They found, like us, that the majority of intratumor blood vessels were of single endothelial cell type and intermediate and only 19% of all tumor blood vessels have perivascular signal for SMA so there are of mature type.

The presence of single endothelial cells and newly formed vessels of intermediate type with high density in neoplastic transformation of the breast tissue could be an indicator of poor prognosis in ductal invasive carcinoma because we found that they were absent in normal mammary gland and fibroadenomas. For agents that do selectively target angiogenic endothelial cells, pericyte and single endothelial cells information may provide additional evidence of therapeutic anti-vascular effect.

Shiozaki K et al. [19] obtained an antiangiogenic chimeric anti-endoglin (CD105) antibody tested with promising results on non-human primates and that had a synergic effect with doxorubicin.

Conclusions

CD 105 (endoglin) represents a useful and more specific endothelial marker that identifies activated endothelium in tumor blood vessels. When used with SMA antibodies in a double immunostaining it makes the difference between immature and mature tumor vessels.

The double immunostaining for CD105/SMA may be used as method for selection of patients susceptible for targeted antiangiogenic therapy.

The differentiation between immature and mature tumor blood vessels using double immunostaining for endothelial and perivascular markers on the same section is an important step for applying an antibody-based antiangiogenic drugs according to the different susceptibility to the therapy of the immature (unstable) and mature (more stable) neovessels.

References

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Received: January 20th, 2007
Accepted: March 20th, 2007