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Heterogeneity of malignant non-Hodgkin lymphoma-associated blood vessels

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Abstract

The present study described for the first time a high heterogeneity of blood vessels in non-Hodgkin lymphomas (nHL). The tumor blood vessels were highlighted with CD105/smooth muscle actin (SMA) and CD34/SMA double immunostaining. For both follicular and diffuse types of lymphomas, more than 85% of CD34/SMA positive vessels were of immature and intermediate type. A percent of 96.54 from CD105/SMA assessed blood vessels were of activated and mature activated types with high expression of CD105 on endothelial cells of newly formed blood vessels. Our results suggest that these types of vessels are potential therapeutic targets for antivascular therapy.

Keywords: non-Hodgkin lymphoma, immunohistochemistry, immature and intermediate blood vessels, angiogenesis.

Introduction

Tumor associated blood vessels are irregular in size, shape, and branching pattern, lack the normal hierarchy, and do not display the recognizable features of arterioles, capillaries, or venules [1]. Intratumoral vessels often show incomplete wall, and therefore, the architecture is abnormal. Intratumoral vessels collapse is frequent, generating hypoxia, followed by necrosis and in these conditions cannot sustain the rapid growth of tumor cells. Tumor vessels specifically express endoglin – CD105, a homodimeric surface component of transforming growth factor (TGF) beta-receptor. Endoglin is highly expressed by endothelial cells of intratumoral vessels but not by pre-existing blood vessels. Therefore, CD105 is useful to discriminate between normal and tumor vessels. First description of tumor blood vessels is dated back almost 30 years and contains nine different classes [2]. More recently, Gee MS *et al.* (2003) [3] have shown that tumor vessels can be segregated into three categories based on size, perfusion and proliferation of endothelial cells, and presence of pericytes: unperfused buds of endothelial cells (immature), intermediate and mature. Because no data was found in the literature that addresses this issue in lymphomas, the purpose of this paper is to describe the types of tumor-associated vessels, their density and possible significance.

Materials and Methods

We have included in our study eighteen specimens of lymph node biopsies from patients with nHL and three normal lymph node specimens as control slides. Fifteen patients were diagnosed with diffuse, B-cell lymphoma type and three patients presented follicular nHL, according to REAL classification. Specimens were fixed in buffer formalin and paraffin embedded. Step sections, 5 μm thick, were performed for each case. Sections from each case were stained with Hematoxylin and Eosin method, for the pathologic diagnosis. For double immunostaining we used CD105, clone SN6h, one hour incubation, dilution 1:100; CD34, QBEnd10 clone, 1:25, 30 minutes and smooth muscle cell actin (SMA), clone 1A4, ready to use, 30 minutes. The Envision double stain kit followed incubation with alkaline phosphatase. All reagents were from Dako, Glostrup, Denmark. The entire immunohistochemical procedure was performed with a DakoCytomation Autostainer (DakoCytomation, Denmark). Tumor blood vessels were quantified separately for CD34/SMA according with Gee MS *et al.* [3] as immature, intermediate and mature and for CD105/SMA (as activated – CD105+/SMA-, mature – CD105-/SMA+ and mature activated – CD105+/SMA+). Blood vessels were counted in the tumor area using the hot spot method, according to the procedure published by Weidner N *et al.* (1992) [4]. Microscopic images were captured as JPEG format, and processed using Nikon Lucia G software (Nikon, Tokyo, Japan).

The local research ethics committee approved the protocol of the study, and informed consent was obtained from all subjects according to the *World Medical Association Declaration of Helsinki*.

Results

Histopathological evaluation based on Hematoxylin and Eosin method revealed 15 cases of diffuse non-

Hodgkin lymphoma and three cases of follicular non-Hodgkin lymphoma.

In the tumor area we identified blood vessels with the following morphological aspects: immature – CD34+/SMA-, without lumen (Figure 1a), intermediate – CD34+, with perfused lumen, but with negative or weak positive reaction for SMA (Figure 1b) and mature tumor blood vessels – CD34+/SMA+ (Figure 1c).

In some cases, we noticed only the presence of immature and intermediate vessels types. The immature vessels, CD34+/SMA-, had a very thin wall, delineate compartments in tumor parenchyma. Some of intermediate vessels contained neoplastic cells in the lumen.

Summarizing, in tumor area of lymphomas, vessels showed a marked polymorphism, characterized by the presence of immature vessels, which expressed only the endothelial marker. We found intermediate vessels, mosaic vessels with focal expression of actin and endothelial cell buds.

The total number of CD34/SMA positive vessels varied between 9 and 53 vessels/field ×200 with an average of 33.89. By CD34/SMA double immunostaining, we noticed immature (74.43%), intermediate (11.13%) and mature tumor blood vessels (14.44%) for both follicular and diffuse type of non-Hodgkin lymphoma.

Double immunostaining CD105/SMA revealed a number of morphological and numerical issues different from the previous one. Thus, preexisting vessels with lumen, regularly expressed only actin and the newly-formed blood vessels from the tumor area expressed only CD105. Focally, we noticed vessels that expressed both markers. This aspect draws attention to the potential of CD105 to signal out activated endothelial cells. Activation phenomenon was found not only in perfused vessels but also to leading endothelial cells from the top of endothelial bud. Endoglin expression was continuously until the intermediate stage of vessels with detectable lumen. As a particular aspect, the entire endothelium of glomeruloid vascular structures expressed endoglin with higher intensity.

For CD105/SMA assessment, the total number of vessels varied between 5 and 49 with an average of 25.1. Mature blood vessels represented 3.46%, activated – 87.18% and mature activated – 9.36%. A percent of 96.54 from the total number of CD105/SMA assessed blood vessels were of activated and mature activated types. The shape, size and particular features of mature (1d), activated (1e), and mature activated (1f) tumor blood vessels are showed in Figure 1.

The highest value for mature activated blood vessels was noticed in a case of follicular non-Hodgkin lymphoma.

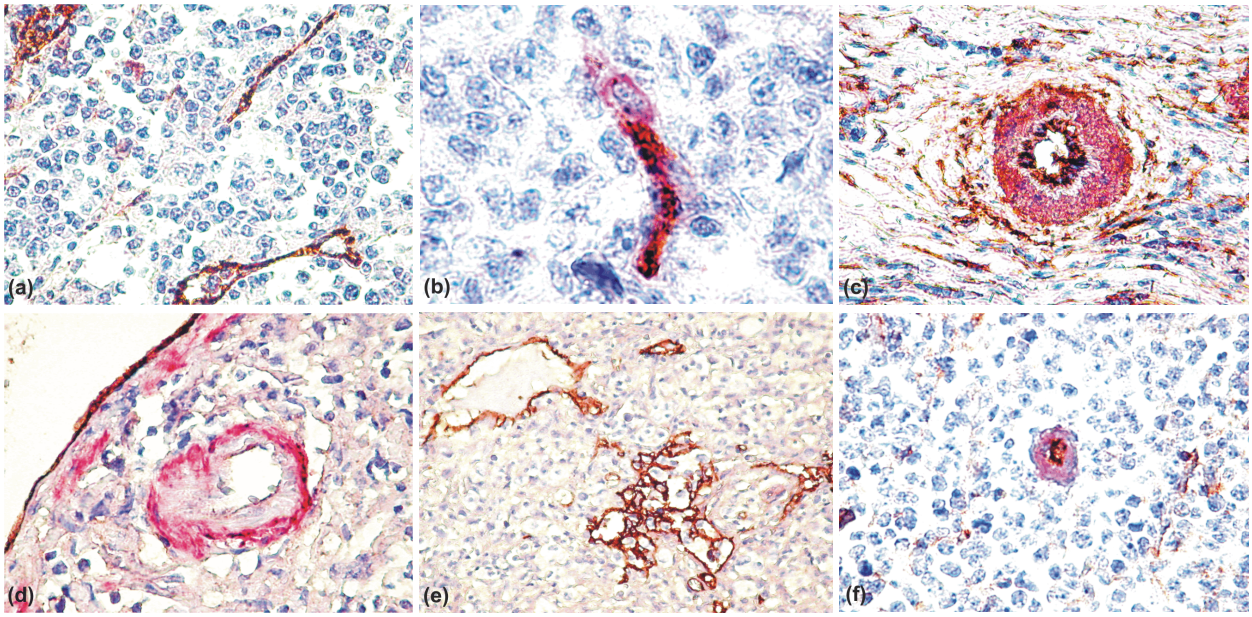

Figure 1 – ***(a) CD34/SMA double immunostaining, ob. ×20, immature vessels. CD34 positive reaction in the endothelium of tumor blood vessels and negative reaction for smooth muscle actin. (b) CD34/SMA double immunostaining, ob. ×40, intermediate vessels. CD34 positive cord like structure and SMA positive cells near an intermediate type of tumor blood vessels. (c) CD34/SMA double immunostaining, ob. ×20, mature vessels. Both markers are positive for mature blood vessels. (d) CD105/SMA double immunostaining, ob. ×20, mature vessels. No positive reaction for endoglin (CD105) in the endothelium of inactive mature tumor blood vessels. (e) CD105/SMA double immunostaining, ob. ×10, activated vessels. CD105 positive vessels inside the lymphoma without expression of SMA. Activated vessels are organized as networks of highly splitted vasculature. (f) CD105/SMA double immunostaining, ob. ×20, mature activated vessels. Tumor blood vessels positive for both CD105 and SMA are probably mature activated vessels, which serve as "mother" vessels for the newly formed blood vessels in lymphomas.***

Discussion

Angiogenesis plays a central role in tumor growth for malignant solid tumors and there is emerging evidence showing that tumor progression of hemato-lymphoid malignancies also depends on the induction of new blood vessel formation [5]. Ribatti D *et al.* [6] reported that microvascular density (MVD) is correlated with the biological behavior in nodal B-cell lymphoma and the frequency of nHL tissue microvessels increases at the same time with the pathological progression.

Also, it has been noticed increased capillary proliferation in the lymph node biopsies of high grade nHL. There was found a significant difference in MVD measured by immunostaining with anti-Factor VIII related antigen between low and high grade nHL classified according to Working Formula classification or Kiel classification [7]. Mazur G *et al.*, found no significant correlation between MVD revealed by CD34 staining and the grade of histological malignancy in the lymph nodes of patients with nHL, based on REAL classification [8].

In our study, more than 85% of CD34/SMA stained vessels were of immature and intermediate type. These findings suggests an active angiogenesis in nHL and could be useful for choosing of a proper antiangiogenic and/or antivascular therapy targeting especially immature and intermediate blood vessels. 96.54% from CD105/SMA assessed vessels were of activated and mature activated types with high expression of CD105 on endothelial cells of newly formed blood vessels.

The higher percent of CD105 positive blood vessels compared with CD34/SMA assessment showed that the use of CD 105 for differential counting of tumor blood vessels in lymphomas is more sensitive and can appreciate in a more accurate fashion angiogenic status of nHL, aspect already described for other tumor types (including angioimmunoblastic lymphomas) with a stronger impact on therapeutic approach [9–11]. CD105 represents a potential target for anti-CD105 monoclonal antibody therapy, already applied in phase I clinical trials in patients with advanced refractory solid tumors but not yet in lymphomas [12, 13].

Differential counting of immature and mature tumor blood vessels by using double immunostaining for CD34 and smooth muscle actin, correlated with their classification into activated and non/activated vessels by the presence of CD105 positivity can be associated with the degree of metastasis from nHL because of their higher potential to favor passing of tumor cells into bloodstream and subsequent tumor spreading. Metastasis experimental models described CD105 involvement in lung metastasis from breast cancer [14]. Our data, concerning the predominance of CD34+/SMAct-immature, CD105 activated and CD105 mature activated blood vessels in nHL support the high metastatic behavior specific for this type of lymphomas. Highly permeable immature, activated blood vessels observed in our study could sustain a rapid dissemination of malignant cells through bloodstream pathway, adjacent to lymphovascular invasion.

This hypothesis is also sustained by the use of immunotoxins and radioimmunoconjugates generated with anti-CD105 monoclonal antibodies (mAbs) which can inhibit angiogenesis and prevent the growth and metastasis of cancerous tumors [15]. A novel diagnostic and therapeutic method based on CD105 presence in nHL blood vessels could improve diagnostic accuracy and evaluation of the disease concerning its spreading throughout the body. We consider that only immature and intermediate vessels could be seen as potential targets for antivascular specific targeted therapy in non-Hodgkin lymphomas.

Several mechanisms of blood vessels activation were discussed before. Involvement of mast cells in tumor angiogenesis from nHL was previously studied by our group [16]. On our previous paper, we reported the role of mast cells in activation of nHL angiogenesis, based on a significant correlation between the high number of mast cells and newly formed blood vessels. However, this study lacked the differential correlation of mast cells with activated versus non-activated nHL tumor blood vessels. Further studies will be needed to elucidate the specific role of mast cells in the activation of tumor blood vessels. Also, few data reported changes in CD34+ vascular area in nHL blood vessels assessed by morphometry [17] but none of them did report differential assessment of vascular area for immature or mature blood vessels, nor for activated *vs.* non-activated.

Conclusions

Evaluation of blood vessels from non-Hodgkin lymphoma, highlighted with CD34/SMA and CD105/SMA double immunostaining suggests the predominance of immature and intermediate type blood vessels as well as activated and mature activated blood vessels in both type of lymphomas compared with the presence of mature blood vessels, which were more numerous in normal lymph nodes.

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