Angiogenesis in the human thymoma assessed by subclassification of tumor-associated blood vessels and endothelial cells proliferation

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Abstract
The prognostic value of tumor-associated angiogenesis is still a subject of debate. As microvascular density and the expression of different growth factors were not demonstrated to be good predictors of the response to antiangiogenic and antivascular therapy, there is a strong need to search for more sensitive markers. In the present study we evaluated by double immunohistochemical staining the profile of tumor-associated blood vessels and the rate of endothelial cell proliferation in patients with thymoma (n=38). Results were compared with specimens of normal thymus and from patients with myasthenia gravis. We found a significant increase in the number of immature and intermediate blood vessels in the tumor area of thymoma, regardless the histological type of the tumor. Proliferating endothelial cells were found in 15 cases, and co-expression of Ki67 and CD34 had the highest value in immature vessels. Both blood vessel type and endothelial cell proliferation significantly correlated with invasive thymoma. Based on these findings, it can be assumed that the type of tumor-associated vessel together with endothelial cell proliferation are useful predictors of invasion, immature and intermediate vessels can be targeted with antivascular drugs and endothelial cell proliferation could be used as a good predictor of the response to anangiogenic therapy.

Keywords: angiogenesis, endothelial cells, human thymus, immunohistochemistry, prognosis, thymoma.

Introduction
The thymus was extensively investigated in the last hundred years in terms of morphological and immune features. The vast majority of data available about this organ come from experimental models and relatively few authors investigated the human thymus. Although the contribution of the thymus in the development of the immune system is well documented, there are many aspects that are under debate, controversial or even unknown in both normal and pathological conditions [1]. These include normal development in human, the endocrine function, histogenesis of thymoma and the molecular profile of these particular tumors of the mediastinum.

The term thymoma is restricted to the neoplastic proliferation of epithelial cells that form the stroma of the thymus. During the last four decades, many teams tried to classify thymoma based on morphological grounds and there were characterized many types according to the organotypic differentiation and the subtype of epithelial cells of the normal thymus wherefrom these tumors are thought to arise [2, 3]. Even so, the classification of thymoma remains the most elusive from the entire pathology and its impact in prognosis and natural behavior of the tumor is minor. In part, this is due to the small series of patients evaluated by separate teams and nowadays there is a strong need to characterize new predictive markers and eventually, new therapeutic targets. For many years, the main target in cancer research was the tumor cell, but now there is a general agreement that tumor microenvironment plays a crucial role in progression and metastasis.

Tumor angiogenesis is a complex process that leads to the formation of new blood vessels within and around the tumor, favoring invasion and distant spread of malignant cells. The prognostic value of tumor-associated angiogenesis was first shown on the base of microvessel density, but results are controversial in many human tumors and virtually, only two articles were published on this topic in patients with thymoma [4, 5]. As in normal conditions, in malignant tumors angiogenesis is governed by the sequential effect of some growth factors, like vascular endothelial growth factors (VEGF), platelet derived growth factors or fibroblast growth factors. Few data are available about the expression of the growth factors and their cognate receptors in human thymoma, but recently it was shown their differential expression in different types [6, 7].

There were accumulated a lot of data that support the use of antivascular and antiangiogenic therapy in many human malignancy. The favorite target of antiangiogenic therapy is VEGF, and the monoclonal humanized antibody bevacizumab was already introduced in the therapeutic protocols of colorectal, breast or pancreatic cancer. In the large majority of clinical trials, it was used only one antiangiogenic agent,
and this could explain the clinical response that is inferior to expectations based on experimental models. Currently, in thymoma there are ongoing only two clinical trials based on antiangiogenic, but not antivascular therapy, without significant benefits [8].

On the other hand, there are no sensitive and specific enough markers to evaluate the effects of the antiangiogenic therapy on both tumors cells and tumor vasculature. Microvessel density was found to be not suitable for this purpose, as it reflects only the intercapillary distance and includes all the vessels shown with a pan-endothelial marker. Gee MG et al. [9] classified tumor-associated vessels as immature, intermediate and mature, based on the perfused lumen and presence of perivascular cells. This classification was not previously applied to the thymoma vasculature, and this aspect could be important for therapeutic reasons. Only scattered data are available about the rate of proliferation of endothelial cells in the tumor area, and virtually, there is only one publication about this topic in thymoma [10]. Based on the general consensus on the microscopic evaluation of angiogenesis, it seems that endothelial cells proliferation is the most accurate marker that predicts the response to antiangiogenic and antivascular therapy.

In the present study, we investigated the type and incidence of tumor-associated blood vessels, combined with detection of endothelial cells proliferation by double immunohistochemical reaction. The findings were correlated with tumor stage and pathological form in order to assess their predictive value.

**Material and Methods**

There were investigated 38 cases with thymoma classified from pathological point of view according to the requirements of World Health Organization [2]. Additionally, there were included in the study five cases with normal thymus surgically removed from children with cardiovascular malformations, aged between two and five years. Specimens were fixed in buffer formalin and embedded in paraffin according to the standard histological procedure. Five-micrometer thick sections were stained with the routine Hematoxylin–Eosin histological procedure. Five-micrometer thick sections were prepared for immunohistochemistry, as follows: antigen retrieval was performed at microwave in citrate buffer pH 6 using PT link module; endogenous peroxidase was inhibited with 3% hydrogen peroxide, incubation with primary antibody and than application of the LSAB system. Finally, the product of reaction was made evident in brown for the first antibody with aminoethyl carbazole. For the double staining, we used two combinations to evaluate the vessels’ type and the rate of proliferation: CD34 (clone QBend10, ready-to-use)/smooth muscle cell actin (ready-to-use, clone 1A4), and Ki67 (clone MIB1, ready-to-use)/CD34. All reagents used in this study were from DakoCytomation (Dako, Denmark) and the full immunohistochemical procedure was performed with Dako Autostainer Plus (DakoCytomation, Carpinteria, USA).

We evaluated the presence, distribution and number of immature, intermediate and mature vessels on slides stained with CD34/smooth muscle cell actin, and counting was performed to assess microvessel density at magnification ×200. On slides stained with Ki67/CD34 we selectively counted cells showing co-expression of both markers, excluding tumor cells, in three fields from the tumor and peritumoral areas at magnification ×400. The arithmetical media was the final result. The statistic was performed with the commercially available SPSS17.0, and applying Student t-test, p<0.05 was considered as significant.

**Results**

On routine examination, we found the following distribution of the cases, based on the criteria accepted by World Health Organization: type A four cases, type AB five, type B1 seven, type B2 11, type B3 seven, and thymic carcinoma in four of the cases. From 38 cases included in the present study, 23 showed definite invasion of the capsule and surrounding connective tissue.

In the normal thymus and thymus from patients with myasthenia gravis we found that the large majority of blood vessels are of mature type, using the CD34/smooth muscle cells actin double staining (Figure 1).

![Figure 1 – Mature blood vessels in the normal thymus. CD34-positive endothelium is shown in brown and actin-positive perivascular cells in red. CD34/smooth muscle cell actin double staining, ×400.](image)

No immature blood vessels were found in the normal thymus and intermediate vessels were rare, restricted to the junction between cortex and medulla. The average microvessel density (MVD) values for the normal thymus were 1.8/×200 for intermediate and 8.1/×200 for mature blood vessels. In specimens from patients with myasthenia gravis, immature vessels were very rare and we found a slight increase in the number of intermediate vessels in the area of follicular hyperplasia. The average MVD values based on the vessels’ type were as follows: 1.4 immature, 3.2 intermediate and 14.2 mature.

In thymoma we found a significant change in the spectrum of blood vessels. We noticed a marked increase in the overall number of vessels in comparison with the normal thymus and thymus from patients with myasthenia gravis. In the peritumoral area over 90% of
the blood vessels were of mature type. In contrast, in the tumor area the majority of vessels were intermediate, with lumen but without perivascular cells, and immature without a definite lumen and perivascular cells (Figure 2). Only scattered vessels showed perivascular coverage stained in red with anti-smooth muscle cell actin. On occasion, we found vessels with mix features, between intermediate and mature, showing only few perivascular cells (Figure 3).

Counting of blood vessels in patients with thymoma showed in the perivascular area the predominance of mature vessels that ranged between 11.5 and 18.6/×200. The rate of different vessels’ type was 92% for mature, 5.6% for intermediate and only 2.3% for immature vessels.

A dramatic change in this spectrum was found in the tumor area, where we noticed a significant increase in the number of immature vessels (average 7.1) and immature vessels (average 6.8), in comparison with mature vessels (average 3.7). Data on the vessel type-associated MVD are shown in Figure 4. No significant correlation was found between immature and intermediate vessels and invasion (p<0.0031).

Then we evaluated the rate of proliferation of endothelial cells on slides stained for Ki67 and CD34. The expression of Ki67 was found in tumor cells of all the cases with thymoma, with the highest rate in thymic carcinoma. On the other hand, tumor cells did not express CD34, and therefore, we used the co-expression of Ki67 and CD34 to evaluate the endothelial rate of proliferation. For this purpose, we evaluated the endothelial proliferation only in the tumor area, based on previous findings that showed the presence of all three vessels types.

In the mature blood vessels the endothelium was positive for CD34, but not for Ki67 (Figure 5).

Ki67-positive endothelial cells co-expressing CD34 were found in 15 from 38 cases. Co-expression of these two markers was found in both immature and intermediate vessels. The number of endothelial cell-positive nuclei was significantly higher in immature in comparison with intermediate vessels (0.8/vessel vs. 3.1/vessel). In some immature vessels, almost all endothelial cells were labeled for Ki67 (Figure 6).
The expression of Ki67 and CD34 in immature and intermediate vessels is shown in Figures 6 and 7.

No significant correlation was found between the presence and incidence of proliferative endothelial cells and the histological type of thymoma according to the WHO classification, but a strong correlation was found with the invasive character of the tumor \( (p<0.0001) \). We found proliferative endothelial cells in only two cases with non-invasive thymoma and this could suggest its potential value to predict invasion.

**Discussion**

Angiogenesis is the process of new blood vessels' formation from preexisting, associated to synthesis of some particular agents by endothelial cells, as well as the accumulation of some specific mediators in the extracellular matrix [11]. Many authors investigated microvessel density that is thought to be an important prognostic factor in carcinoma with different locations and in other human malignancies as well [12]. Data on this topic in patients with thymoma are very rare in the literature, and features related to the vessel type and endothelial proliferation are lacking. Results of the relationships between MVD and prognosis were often controversial, even for the same tumor type. A possible cause for these controversies could be the counting of all vessels highlighted with a general endothelial marker. Moreover, MVD cannot be used as an indicator of the antiangiogenic therapy, as it only reflects the intercapillary distance. Most probably, better results can be obtained counting only immature and intermediate vessels that actually represent the main target for the antivascular therapy. In the present study, we took into account also the non-perfused vessels that are excluded by some authors, but accepted by others [13]. Nowadays it is considered that MVD calculated on the base of a marker of endothelial cells activation is more valuable for the prognosis in individual patients [14].

There are major differences between normal and tumor-associated blood vessels. Tumor-associated vessels are frequently irregular, with thin wall and abnormal architecture. The collapse of vessels from the tumor area is frequent and generates hypoxia, and consequently, a more rapid growth of endothelial cells. Based on the expression of an endothelial marker and a perivascular cell marker, tumor-associated blood vessels can be classified as mature, immature and intermediate [9]. The distribution and significance of these three types was investigated in few human tumors, like oral squamous cell carcinoma [15], but not in thymoma. We have shown in the present study a significant increase in the number of immature and intermediate vessels in the tumor area of thymoma in comparison with the normal thymus and thymoma. We also found a significant correlation between these vessels and invasion, but not with the pathological type. On one hand, our results support the prognostic value of MVD calculated on slides stained for CD34 and actin, and on the other detects the potential responders to the anti-vascular therapy. Our results are in accord with those published by others in patients with prostate cancer, in which there were identified only 19% mature vessels [16]. To the best of our knowledge, the particular distribution of the vessels’ type in the tumor area of thymoma was not previously reported.

Studies on the co-expression of Ki67 and CD34 are very rare in the literature, and there were not extended to patients with thymoma. Until now, convincing results were published only in the squamous cell neoplasia of the uterine cervix [17] and tumors of the head and neck [15]. In a recent study, it was shown that activation and proliferation of endothelial cells are successive and not coincident steps of tumor angiogenesis [18]. In order to avoid over- or underestimation of the number of Ki67-positive nuclei, a careful computerized analysis of the microscopic image must be used, as also shown by others [19].

In the present study, we found an increased rate of proliferation of endothelial cells in intermediate and mainly in immature blood vessels. We found no significant endothelial proliferation in the case of the normal thymus and myasthenia gravis. This finding supports the hypothesis that in patients with myasthenia gravis the increase of MVD could be due mainly to intussusception and in thymoma mainly to sprouting from preexisting vessels. Our results are in accord with those obtained by others in tumors of the head and neck [15, 20].
Currently, it is largely accepted that there are no valuable indicators of the response to antiangiogenic therapy. Nor the value of MVD, neither the expression of different growth factors was found to be predictive for this purpose. Moreover, there is a lack of experimental models in this field. Our results support the introduction of endothelial cell proliferation as marker on one hand of invasion, and on the other, to predict the response to the antivascular therapy.

Conclusions

In summary, we have demonstrated a significant increase in the number of immature and intermediate blood vessels in the tumor area in patients with thymoma. This finding supports the introduction of antivascular therapy in these patients. The rate of endothelial cell proliferation is higher in immature and intermediate vessels, and this aspect may be useful to predict the response to the antiangiogenic therapy. For both vessels’ type and endothelial cell proliferation, we found no correlation with the pathological form but a significant correlation with invasion.

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