Assessment of angiogenesis in soft-tissue tumors

VIRGINIA BANETH\textsuperscript{1)}, M. RAICA\textsuperscript{2)}, ANCA MARIA CÎMPEAN\textsuperscript{2)}

\textsuperscript{1)Department of Pathology, Emergency County Hospital of Arad
\textsuperscript{2)Department of Cytology and Histology, "Victor Babeş" University of Medicine and Pharmacy, Timișoara}

Abstract

The aims of our paper were to establish the main morphological and immunohistochemical aspects of tumor vessels, to quantify angiogenesis in soft-tissue tumors and to study the possible relationship between mast cells and angiogenesis. For this purpose, we immunohistochemically investigated 54 cases of benign and malignant tumors for smooth muscle actin and endothelial markers CD34 and CD31. It is presented a simple method to demonstrate simultaneously blood vessels and mast cells in the same section, using anti-CD34 and alcian blue-safranin. Our results strongly support the presence of a large number of immature and intermediate blood vessels in the tumor area. Microvessel density was higher especially in liposarcoma and malignant fibrous histiocytoma, and this finding suggests the application of the antiangiogenic therapy in soft tissue tumors in addition to conventional methods.

Keywords: soft tissue tumor, microvessel density, mast cell density, immunohistochemistry, histochemistry.

\section*{Introduction}

The growth of blood vessels is essential for organ growth and repair. An imbalance in this process contributes to numerous malignant, inflammatory, ischemic, infectious and immune disorders. Formation of new blood vessels from preexisting ones (a process known as angiogenesis) is an absolute requirement for the growth, maintenance, and metastasis of most solid tumors [1, 2].

Angiogenic molecules released by tumor and stromal cells mediate this process. The newly formed endothelial cells can stimulate further tumor growth in a paracrine or cell contact mediated fashion [2].

Distinct biological features expressed by tumor-associated vasculature may serve as prognostic markers of disease progression as well as targets for therapeutic intervention. Numerous reports have demonstrated that vascularization assessed by intra-tumor microvessel density (MVD) correlates with clinical and pathological factors and patient prognosis in a variety of tumors [3–6].

In contrast to epithelial tumors and hematological malignancies, few data are available regarding angiogenesis in soft-tissue tumors, its relevance and its prognostic impact.

The genuine role of mast cells in tumor stoma has been a very controversial topic and needs further clarification. Evidence indicates that mast cells play an important role in tumor progression via promoting angiogenesis due to mostly tryptase [7].

There are also data suggesting that increased mast cell density correlates with high microvessel counts and indicates bad prognosis [7–9].

Other, however postulate them to be inhibitors of tumor development through their pro-apoptotic and – necrolytic granules (granzymes and TNF-α) [10].

Other authors consider that a high content of mast cells is a favorable prognostic factor [11, 12].

Recent literature indicates that mast cells could either promote or inhibit tumor growth depending on their phenotype and on the local stromal conditions [13].

The present study was conducted to investigate the extent of blood vessels in soft-tissue tumors, to establish the main morphological and immunohistochemical characteristics of tumor microvessels and to study the possible relationship between mast cells and angiogenesis.

\section*{Material and methods}

\subsection*{Patients}

Paraffin-embedded surgical specimens from 54 patients with soft-tissue tumors, treated in the County Hospital of Arad, were included in this retrospective study. There were 30 males and 24 females with a median age of 52 (range, 7-77 years) at the time of diagnosis. Forty-three tumors were malignant and 11 were benign proliferations.

\subsection*{Immunohistochemical studies}

Serial sections (5µm-thick) of paraffin-embedded surgical specimens were processed for immunohistochemical identification of microvascular endothelial cells with anti-CD31 (clone JC/70A, prediluted, Dako) and anti-CD34 (clone QBEND10, prediluted, Dako) antibodies.

The anti-smooth muscle actin (clone 1A4, prediluted, Dako) was also used to demonstrate the presence of the vascular wall. Immunohistochemical localization was performed by the labeled streptavidin-biotin technique (Dako-LSAB kit) with a DAKO automated slide-processing instrument.

Before staining tissue sections were dewaxed in xylene, rehydrated in a graded ethanol series, and microwaved for 20 minutes in citrate buffered saline pH6 to enhance antigen retrieval.

The final product of reaction was stained in brown
using diaminobenzidine dihydrochlorid (DAB) as chromogen. Sections were counterstained with modified Lillie haematoxylin.

**Combined immunohistochemical–
histochemical method**

CD34-immunostained sections, after visualization of the final product of reaction with DAB, were counterstained with the alcin blue-safranin method at pH 0.2.

**Microvessel counting**

The degree of angiogenesis was determined by the microvessel density in a defined hot spot area according to the method of Korkolopoulou et al. [6].

The investigators were not aware of the diagnosis before performing the microvessel counting. The CD31-immunostained sections were systematically scanned to find the area showing the most intense vascuularization. Three representative fields at × 200 magnifications were chosen for each tumor. These fields were digitized as JPEG images using a digital camera system (Coolpix 950). Image-processing software (Lucia G, version 6.1 Nikon) and the mean number of microvessels per field (MVD) analyzed the stored digital images and mast cell density (MCD) was calculated.

**Statistics**

Differences in microvessel and mast cell density between malignant and benign tumors and between different sarcoma types were analyzed by the SPSS10.0. Two-sided $P$ values of .05 or less were considered significant.

## Results

The results of the immunoreactions for the endothelial markers CD34 and CD31, reproduced in Table 1, allowed the comparison regarding the sensitivity and specificity of the two antibodies and the calculation of the microvascular density, based on the parallel counting of the blood vessels within the sections stained with anti-CD34 and anti-CD31.

**Table 1 – Intensity of immunohistochemical reactions for endothelial markers**

<table>
<thead>
<tr>
<th>Antibody/intensity</th>
<th>High</th>
<th>Moderate/low</th>
<th>Negative reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD34</td>
<td>51</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>CD31</td>
<td>34</td>
<td>15</td>
<td>5</td>
</tr>
</tbody>
</table>

As part of the interpretation of the results regarding the sensitivity of the methods, it was taking into consideration only the positive immunoreaction at the endothelial level, from the interpretation being excluded the positive tumor cells (for example positive CD34 in dermatofibrosarcoma protubersans). As it can be observed from the table above, the most sensitive reaction has been observed for the antibody anti-CD34, which has been constant and of high intensity for most of the cases.

The immunoreaction for CD31 was also positive for a great number of the cases, but of variable intensity. The results we have obtained point out the fact that the reaction for CD34 has a maximum sensitivity for the identification of the vascular endothelium, but limited specificity, because some of the tumors included in the study were also positive at the level of the neoplastic cells cytoplasm. The immunoreaction for CD31 has a very good sensitivity and higher specificity than CD34, because it was positive only for one case, diagnosed as angiosarcoma.

The morphological and immunohistochemical analysis allowed us to establish the type of the intratumor vessels. Isolated endothelial cells or cords of non-perfused endothelial cells, without lumen, for which the reaction for endothelial marker has been positive, represent the immature vessels. They do not have pericytes or smooth muscle cells in the wall (Figure 1).

The intermediate vessels had usually a large lumen, pointed out with CD34/CD31, but the reaction for smooth muscle actin was negative or focally positive. We have constantly noticed the transition from vessel with negative or focal positive reaction for actin to vessel of larger dimensions, with continuous positive reaction for smooth muscle actin (Figure 2a).

The mature vessels were characterized through the co-expression of endothelial markers and actin, having the significance of stabilization of the newly formed vessels (Figure 2b).

The immunoreaction for smooth muscle actin revealed a particular aspect: for some vessels of mature type with a large lumen, in certain zones, the immunoreaction was abruptly negative, a new lumen being formed at this level. This aspect points out the active tumor angiogenesis, with formation of new immature and intermediate vessels.

By means of comparative analysis of the immunostained sections for CD34 and smooth muscle actin of the same cases, we ascertain that the majority of the blood vessels are of immature and intermediate type and stabilized mature blood vessels represent only 20%. This aspect was even more obvious in cases of liposarcoma and malignant fibrous histiocytoma, and was correlated with the degree of differentiation. The microvessel density (MVD) was appreciated according to the hot spot method, being chosen three fields of maximum vascular density from the tumor area.

The evaluation of MVD was made according to the above-described methodology, on sections immunostained with CD34/CD31. There were taken into consideration only the structures with lumen presenting a positive reaction at endothelial level. The highest values of the MVD were noticed for the cases of liposarcoma and malignant fibrous histiocytoma, aspect concordant with the large number of vessels of immature and intermediate type.

Among the malignant and benign tumors, we noticed the major differences regarding the number of blood vessels. From the statistic point of view, these differences were significant for $p<0.02$. We have also noticed differences between the group of leiomyosarcomas and fibrosarcomas on one hand and liposarcomas and malignant fibrous histiocytomas on the other hand, significant values for $p<0.05$ (Table 2).
Assessment of angiogenesis in soft-tissue tumors

Figure 1 – Immature vessels, without permeable lumen (anti-CD34, ×400)

Figure 2 – (a) Mature vessel with continuous reaction to actin, with transition to immature vessel, with weak or absent reaction; (b) Mature vessels, with pericytes/smooth muscle cells in continuous layer (Anti-actin, DAB)

Figure 3 – (a) Simultaneous identification of blood vessels and mast cells with the combined method anti/CD34-alcian blue-safranin; (b) Safranin type mast cells; (c) Alcian blue positive mast cells
Table 2 – Mast cell density and microvessel density in soft-tissue tumours

<table>
<thead>
<tr>
<th>Tumour type</th>
<th>Peritumoral Intratumoral</th>
<th></th>
<th>MVD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MCD</td>
<td>MCD</td>
<td></td>
</tr>
<tr>
<td>Benign tumours</td>
<td>7.06</td>
<td>6.53</td>
<td>10.3</td>
</tr>
<tr>
<td>Fibrosarcoma</td>
<td>8.2</td>
<td>15.4</td>
<td>34.6</td>
</tr>
<tr>
<td>Liposarcoma</td>
<td>6.9</td>
<td>22.1</td>
<td>61.2</td>
</tr>
<tr>
<td>Malignant fibrous histiocytoma</td>
<td>9.7</td>
<td>31.4</td>
<td>58.9</td>
</tr>
<tr>
<td>Leiomyosarcoma</td>
<td>5.4</td>
<td>16.6</td>
<td>21.5</td>
</tr>
</tbody>
</table>

MCD – mast cell density; MVD – microvessel density

Having in view the preferential perivascular disposition of the mast cells and the potential of these cells to secrete vascular endothelial growth factor, we have investigated the density of these cells in sarcoma and benign tumours. The mast cells have been counted both within the tumor area, and within the peritumor one, using the method of histochemical and immunohistochemical double staining. On the sections stained with this method (whose description we haven’t found in literature), we could identify both the blood vessels, and the mast cells, regardless of their type (Figure 3a).

The endothelium was stained in brown with diaminobenzidine, and the mast cells in red with safranin (Figure 3b) or in blue with alcian blue (Figure 3c). We noticed that the mast cells were present in the proximity of the blood vessels, but also among the tumor cells.

This method allow us to demonstrate that with regard to the values of the peritumor mast cells, there are no significant differences between the benign and malignant tumors but, within the tumor area, differences are significant for p<0.05. Meanwhile, we noticed a direct correlation, non-linear in type, between the number of mast cells within the tumor area and the number of the blood vessels from the same zones (Table 2).

Discussions

Angiogenesis, formation and growth of new blood vessels, plays a key role in tumor development. The intra-tumor newly developing vessels has different morphology and they are not classified according to the general accepted histological criteria. These vessels frequently have a large lumen, a very thin wall, and the endothelial cells often present activation aspects: they have a well represented cytoplasm, the nucleus is not flattened, they have an irregular outline and present discontinuities of the final product of reaction of the specific marker. Due to these aspects, Gee et al. [14] classified the intra-tumor blood vessels as being immature, intermediate and mature.

We have proved that the majority of the blood vessels within the tumor area are of immature and intermediate type. The immature vessels, consisting of highly proliferative, non-perfused endothelial cells sprout positive for CD31/CD34, were the most numerous in the low differentiated cases. The identification of the degree of maturation of the tumor vascular bed points out the malignant tumors potentially responding to the antiangiogenic agents therapy, this kind of therapy acting only on the vessels of immature and intermediate type [14, 15]. Data regarding the microvessel density in sarcomas are confined and mostly diverging. Some authors didn’t find any relationship between the microvessel density and differentiation [16]. Our results don’t confirm these observations, because the highest density has been noticed for the low differentiated cases.

Human sarcomas express a number of proangiogenic factors that may represent therapeutic targets, with vascular endothelial growth factor (VEGF) being the best characterized. VEGF overexpression was demonstrated in many soft tissues sarcomas, with a higher intensity for the lower differentiated cases [17]. In this context, VEGF expression wouldn’t represent an independent prognostic factor of clinical outcome. Yet, other authors point out the predictive character of VEGF expression for pulmonary metastases. The most intense expression of VEGF has been identified in malignant fibrous histiocytoma [18].

These results are according to our observations, which reveal maximum density of blood vessels in malignant fibrous histiocytoma and liposarcoma. Some reports mention overexpression of VEGF in leiomyosarcoma, but the investigations were made on a low number of cases and it is not in agreement with our observations noticing MVD with an intermediary value. The studies regarding angiogenesis in sarcomas are very rare comparing to those published for carcinomas, and therefore new studies performed on large series of patients are necessary.

The mechanisms implicated in the formation of new blood vessels within tumours of soft tissues are even less known, no article regarding this subject being published in the literature. As for other malignancies, tumour cells might be a source of VEGF. Undifferentiated elements can give rise to endothelial cells on one hand, but also circulating progenitors of endothelial cells may be inserted in the pre-existing vessels [19]. The mast cells, often identified by us in sarcomas, certainly have an inductor role, most probable due to the capacity of secretion of VEGF. At present, there are few studies about the relationship between mast cells and angiogenesis in sarcomas, and the proof of their implication in this process might clarify not only the aspects regarding the fundamental mechanisms, but it might also have therapeutic implications through the association of the mast cell anti-degranulants to the antiangiogenic therapy.

Conclusions

The present investigation has demonstrated a significantly higher microvessel density in soft-tissue sarcomas, especially liposarcoma and malignant fibrous histiocytoma, as compared with benign proliferations. Our method for simultaneous identification of blood vessels and mast cells has not been described for quantification of angiogenesis up to now; this fact has allowed us the emphasizing of a significant statistic correlation between the number of mast cells and microvascular density.
These observations strongly support the hypothesis of an important role of angiogenesis in soft-tissue sarcomas. Furthermore, these findings suggest that antiangiogenic therapy could represent a novel strategy for the treatment of soft-tissue malignant tumors, considering the deceiving results of conventionally anti-neoplastic treatment.

References


Mailing address
Marius Raica, Professor, M. D., Ph. D., Department of Cytology and Histology, “Victor Babeș” University of Medicine and Pharmacy, 2 Eftimie Murgu Square, 300041 Timișoara, Romania; Phone / Fax +40256–490 626, E-mail: raica@umft.ro

Received: March 2nd, 2006

Accepted: April 30th, 2006