Angiogenesis in urothelial tumors of the upper urinary tract

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Abstract

Angiogenesis is an essential process in the progression of malignant tumors. Tumors of the ureter and renal pelvis account for 5% of all urinary tract neoplasms. Little is known about angiogenesis in upper urinary tract urothelial tumors. We tried to demonstrate angiogenesis by using three endothelial markers CD31, CD34, von Willebrand factor and one pericytes marker (α-smooth muscle actin) in 26 cases.

The pattern of CD31 immunolabelling was more complex and extensive than the vessel pattern shown by CD34 or factor VIII staining. In non-invasive tumors we observed that angiogenesis process is limited to connective tissue of tumor stroma. In the tumor area, the blood vessels stained with anti-CD31 had large lumen, thin walls and numerous branches, some of them being very thin. Pericyte covered vessels were branching of frequently into smaller, pericyte negative vessels.

Keywords: angiogenesis, endothelial cells, urothelial tumors, blood vessels, immunohistochemistry.

Introduction

Angiogenesis involves extracellular matrix remodeling, associated with proliferation and migration of endothelial cells [1].

During vasculogenesis, the precursor cells differentiate into endothelial cells, which assemble into first vascular networks [2].

During embryonic and fetal development, angiogenesis and vasculogenesis occur concomitantly.

In 1971, Folkman popularized the concept of tumor angiogenesis [3], and demonstrated that angiogenesis was essential for the progression of the malignant tumors.

Solid tumors could not grow beyond two mm in diameter without a generation of new vessels [4].

Tumors of the ureter and renal pelvis account for 5% of all urinary tract neoplasms and of these greater of 90% are urothelial carcinomas [4].

The incidence of these tumors has increased slightly in the last thirty years being more common in older patients.

Because of the reduced incidence of patients with such tumors, relatively few data are available about their natural evolution and behavior.

The morphological and immunohistochemical profile overlaps only in part with those reported for similar tumors developed in the urinary bladder, which, in their turn, are well known nowadays.

In our region, the incidence of ureter and renal pelvis tumors is higher, reaching more than 9%.

Therefore the main objective of the present study was to determine the light microscopic and immunohistochemical characteristics of the angiogenesis in urothelial tumors.

Material and methods

Specimens

We retrospectively investigated 26 patients with urothelial tumors of the upper urinary tract, aged between 47 and 68 years, admitted in the Clinical Hospital of Timișoara.

The surgical technique performed in all patients was nephroureterectomy, so in each case specimens were taken from the tumor, kidney and ureter.

Specimens were fixed in 4% buffered formalin, embedded in paraffin, and step sections were stained with routine Haematoxylin–Eosin method.

Immunohistochemistry

Additional slides were immunohistochemically stained, using the three steps labeled streptavidin-biotin-immunoperoxidase technique (LSAB2, DAKO, Glostrup, Denmark).

After endogenous peroxidase inhibition, antigen retrieval, the sections were incubated with the first step antibodies: anti-factor VIII (von Willebrand, monoclonal, clone F8/86), anti-CD31 (monoclonal, clone JC70A) and anti-CD34 (monoclonal, clone QBEnd10).

The pericytes were identified with anti-α-smooth muscle actin antibody (monoclonal, clone 1A4). Thereafter, the slides were washed in PBS and consecutively reacted with a labeled streptavidin-biotin system. The reaction product was visualized in brown with DAB as chromogen. The nuclei were counterstained with Mayer’s Haematoxylin.
cells and megacaryocytes and mediate platelet adhesion and thrombus formation at the sites of vascular injury.

CD31 (PECAM) is a glycoprotein found on various hematopoietic cells such as bone marrow stem cells, CD8+ cells, monocytes, neutrophils, platelets and endothelial cells. This molecule is involved in cell adhesion and mediates the transmigration of leucocytes from blood vessel lumen into the extravascular space.

CD34 is a transmembrane glycoprotein present on hematopoietic stem cells and endothelial cells.

Actin is a protein, which presents at least six different isotypes. Ultrastructurally, actin forms diffusely distributed cytoplasmic 6 nm microfilaments. The distribution of α-smooth muscle actin is restricted to pericytes, vascular media, smooth muscle of viscera and myoepithelial cells of salivary gland and breast.

Anti-vimentin (clone V9) antibody was used as a marker of the optimal fixation and embedding procedures. The external positive control was represented by slides from a capillary haemangioma. As internal positive control, we used immunolabelling of endothelial glomerular cells. As negative control, it was used a non-specific immunoglobulin, provided by the manufacturer (DAKO, Denmark), performed on slides from the same cases. All reagents for the immunohistochemical technique were supplied from DAKO, Glostrup, Denmark.

Results

We examined blood vessels in urothelial tumors of upper urinary tract. The site of primary tumor is shown in Table 1.

Table 1 – The site of primary tumor

<table>
<thead>
<tr>
<th>Tumor location</th>
<th>No. of cases</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calyces</td>
<td>4</td>
<td>15</td>
</tr>
<tr>
<td>Renal pelvis</td>
<td>5</td>
<td>19</td>
</tr>
<tr>
<td>Calyces + Renal pelvis</td>
<td>7</td>
<td>27</td>
</tr>
<tr>
<td>Ureter</td>
<td>8</td>
<td>31</td>
</tr>
<tr>
<td>Renal pelvis + Ureter</td>
<td>2</td>
<td>8</td>
</tr>
</tbody>
</table>

We studied the morphological aspects of angiogenesis in urothelial tumors of upper urinary tract. There were a lot of differences between cases with the same stage and grade. The sprouting of new vessels from preexisting vessels could be observed with morphological staining such as Hematoxylin–Eosin (Figure 1).

Even if some blood vessels could be evaluated on Hematoxylin–Eosin stained slides, morphological methods underestimated the number of blood vessels with at least 30%. Moreover, morphological methods could not highlight the presence of single endothelial cells, without lumen, which preceded the canalization phase of new blood vessels formed during angiogenesis (Figure 2).

That’s why we evaluated the genesis of new blood vessels using the immunohistochemical methods. The first intention was to establish the sensitivity and specificity of the immunohistochemical methods in the urothelial carcinoma. The examination of thin (5 µm) tumor sections stained with anti-von Willebrand factor revealed that the tumor vessels varied greatly in size. The small caliber vessels were predominating.

At the smallest end of some vessels there were isolated cells positive for anti-factor VIII related antigen, lacking a visible lumen. These cells stained also for other endothelial cells markers as CD31 and CD34.

The pattern of CD31 immunolabelling was more complex and extensive than the vessel pattern shown by CD34 staining. Therefore, the vessels localization and morphology were based on results obtained with these two antibodies. We found that factor VIII related antigen is not enough sensitive for proper counting, because usually it underestimates the mean value of blood vessels.

On CD31 immunolabelled slides we observed the largest number of endothelial cells positive for this antibody. In one case, there were observed thin sprout-like filaments without a lumen CD31 positive, which radiated from the vessel wall into the perivascular space of tumor (Figure 3).

The tumor vessels were characterized with respect to pericyte coverage, by staining with anti-α-smooth muscle actin antibody (SMA). Pericyte positive vessels were defined as CD34 positive structures associated with at least one SMA positive cell (Figure 4).

In non-invasive tumors (stage Ta or carcinoma in situ) the blood vessels were sparsely distributed in tumor area. In the connective tissue of tumor, the blood vessels number has been increased in cases associated with presence of inflammatory cells.

In most cases, the blood vessels of the tumor area had a large lumen, thin walls and numerous branches, some of them being very thin. The larger blood vessels consisting of endothelial cells and pericytes were branching into smaller, pericyte negative vessels, formed only by endothelial cells. These aspects cannot be observed in the connective tissue from normal urothelial mucosa.

On one slide, in the tumor area there were observed small clusters of endothelial cells surrounded by single endothelial cells, a characteristic aspect for the first stage of angiogenesis. The presence of single endothelial cells suggested the progression of tumor angiogenesis. The presence of intravascular embolus could be demonstrated in some vessels using these immunohistochemical methods (Figure 5).

We observed few vessels in cases with extensive necrosis. A very important issue for tumor progression was represented by the proliferation of newborn blood vessels. The vascular sprouts arose from blood vessels present in the connective tissue. First we observed the proliferation of single endothelial cells followed by tube formation.

While the blood vessels expanded, they invaded the connective tissue of tumor stroma until they reached the stroma-epithelium interface (Figures 6 and 7).

Then the blood vessels invaded the tumor parenchyma (Figure 8).
Figure 1 – Small blood vessel of capillary type with sprouting toward malignant cells

Figure 2 – Blood vessels with large lumen, thin wall and many branches in the tumor stroma

Figure 3 – CD31 positive sprouts made visible by LSAB-DAB immunohistochemistry extend from the vessel surface into the tumor

Figure 4 – Pericyte positive vessels stained with anti-SMA antibody
Figure 5 – Unstained tumor cells observed within a blood vessel lumen lined by endothelial cells, stained for CD31.

Figure 6 – Newborn blood vessels in connective tissue of urothelial carcinoma.

Figure 7 – The blood vessels at stroma–epithelium interface.

Figure 8 – Small blood vessels and single endothelial cells invading the tumor parenchyma.
On the same slide, depending of site, there could be revealed different aspects of angiogenesis regarding the number and the distribution of newborn blood vessels. The blood vessels are sparse and have bigger lumen in connective tissue of sites with urothelial dysplasia near by tumor carcinoma. In the connective tissue of carcinoma, the blood vessels are frequent and have thin lumen.

**Discussions**

Angiogenesis occur mainly during embryonic development and in postnatal tissue growth and differentiation. In adults, angiogenesis takes place mainly in the female reproductive system during the menstrual cycle and in pregnancy. Angiogenesis begins from preexistent vasculature, being either the primitive vascular plexuses formed by vasculogenesis in the embryo or the postcapillary venous compartment of the mature vascular systems. The endpoint is an elaborate vascular network that meets the nutritional and functional demands of the respective organ [5].

Pathologic angiogenesis takes place in numerous diseases such as cancer, diabetic retinopathy, age-related macula degeneration, rheumatoid arthritis, psoriasis, inflammatory bowel diseases, endometriosis and many more. The neovascularization is regarded to be causative for the detrimental effects of some diseases and to take part in progression of others. Common characteristics of pathological neovascularization include abnormal vascular permeability and defective vascular remodeling and maturation, which promote leakage, hemorrhaging and inflammation [5].

At least two different types of angiogenesis are known: sprouting of capillaries from preexisting vessels and non-sprouting angiogenesis or intussusceptions [6, 7].

The early phase of angiogenesis consisted in increase of capillary permeability. The normally highly differentiated, flattened endothelial cells and adjacent pericytes become activated, displaying hypertrophy and an increased number of organelles [8].

Then, sprouting angiogenesis implies proteolytic degradation of basement membrane and proliferation and migration of endothelial cells through the extracellular matrix. Both pericytes and endothelial cells contribute to protease degradation of basement membrane in the angiogenic process. After formation of vascular sprouts, the next phase of angiogenesis involves tube formation with final connection of the blind ending sprout to another blood vessel.

In contrast to sprouting angiogenesis, which is a well established mode of blood vessel formation, intussusceptive angiogenesis is a relatively rare concept in vascular biology. It was first discovered in the lung as a means capillary network growth [9].

Intussusception causes growth of the capillaries by insertion of columns of cells or interstitial tissue inside the vessel lumen followed by the vessels splitting into multiple vessels.

In our study, on slides from patients with non-invasive tumors we observed that angiogenesis process is limited to connective tissue of tumor stroma. On our slides, using immunolabelling we were able to demonstrate different phases of angiogenesis in invasive tumors. The immunolabelling of endothelial cells with anti-CD31 antibody was cytoplasmic. Comparing the results obtained for each case, on slides stained with methods mentioned above, we may conclude that the highest sensibility is provided by CD34 and the highest specificity by CD31. Immunohistochemical stains using endothelial markers labeled the vessels sprouts from the early phase of angiogenesis.

There have been described some majors differences between normal blood vessels and intratumoral vessels. Tumor blood vessels have multiple structural and functional abnormalities. Their unusual leakiness, potential for rapid growth and remodeling, and expression of distinctive surface molecules are not only responsible for mediating hematogenous spread of tumor cells and maintaining the unusual microenvironment of tumors but also are key to the efficacy of targeted tumor therapy [10, 11].

There have been described three different types of tumor vessels: least mature, intermediate and mature [12].

The least mature vessels were nonperfused endothelial cells sprouts emanating from functional vessels, the intermediate blood vessels were small, perfused but not covered by pericytes. The mature vessels were large, pericytes covered with quiescent endothelial cells and few associated sprouts. The majority of tumor vessels observed on our slides were intermediate or immature. Many of the intratumoral vessels were collapsed.

In non-invasive tumor of the upper urinary tract, the angiogenesis was limited to the tumor stroma. The walls of most intratumoral vessels were immature, consisted only of endothelial cells and comparing with normal blood vessels presented morphological differences.

In T2 stage urothelial tumors, there were observed microvessels with large lumen, feature important for the metastatic process.

Larger tumors tend to have a lower density of newborn blood vessels and a higher number of mature vessels suggesting that the morphology of tumor blood vessels changes as tumors enlarge.

The vasculature in many tumors was not covered by pericytes, which reflected functional immaturity of tumor vascular bed.

**Conclusions**

Tumor angiogenesis can be detected by immunohistochemical staining of endothelium. Vascular endothelium had a heterogeneous antigenic profile, expressing a number of molecules such as CD31, CD34 and von Willebrand factor, considered endothelial cells markers.
Each of the three antibodies used to identify endothelial cells gave a different staining when tested on different tumors of the upper urinary tract. Between the labeling methods that we used, the highest sensitivity was shown by CD34 immunolabelling and the highest specificity by CD31 immunolabelling.

Therefore we recommend studying the presence of angiogenesis using a panel consisting of all three antibodies (anti-CD31, anti-CD34 and anti-factor VIII related antigen).

Our study highlights vessel development in urothelial tumor. We show that tumor vessels develop through identifiable stages, beginning with lines of endothelial cells emanating from blood vessels. The presence of angiogenic sprouts suggests the tumor angiogenic activity.

These proliferate endothelial sprouts evolve into small caliber tumor vessel, consisted of perfused endothelial tubes lacking pericytes. In later stages of angiogenesis, perfused endothelial tubes gain pericytes coverage, by contiguous spread. The blood vessels with pericytes coverage had large caliber and reduced sprouting.

A tumor response to anti-angiogenic therapy will be primary influenced by the tumor vessels morphology. Because of this, pericyte coverage and protection of tumor vessels may strongly influence tumor therapeutic response. A better understanding of vessel development and maturation in urothelial tumor and of the factors affecting pericyte coverage of their vessels may advance anti-angiogenic therapy of urothelial tumors.

References


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