Immuno-Morphometric Study of Choroid Melanoma Angiogenesis

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Introduction: The lack of local epidemiologic data, and the progress of current diagnosis and treatment methods motivated us to evaluate our patients from the perspective of tumor vasculogenesis. The metastatic potential of the choroidal melanoma is well known, and the vasculogenesis is a promoter of it, making early diagnosis and treatment a necessity.
Material and method: In this paper we present 21 cases of choroidal malignant melanoma from 2005 to 2009. For all the patients the treatment was the enucleation of the eye, followed by histopathologic examination and imunohistochimic staining. Tumor vasculature was followed with immune-staining: CD31, CD105, SMA, Collagen IV. The results were digitalized and analyzed with the ImageJ software to demonstrate vasculogenesis.
Results: Vessels with CD31 positivity were dominant at the periphery, while CD105 positive new vessels were predominant centrally in the tumor mass. Collagen IV staining presented fragmentation and pluristratification of the vascular basal membranes, and the vascular smooth muscle was barely noticeable in the central areas.
Conclusions: The lesion of the vascular wall is evident, signaling the modification of the structure and proportion of the elements. The difference between the peripheral and central area is evident, well documented immunohistochemically and morphometrically. The differences are statistically significant.

Keywords: choroidal melanoma, angiogenesis, immune-morphometry

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Introduction

The lack of local epidemiologic data, the progress of current diagnosis and treatment methods motivated us to evaluate our patients from the perspective of tumor angiogenesis. The metastatic potential of the choroidal melanoma is well known, and the angiogenesis is an essential promoter of it, making early diagnosis and treatment a necessity [1].

Material and methods

In this paper we present 21 cases of choroid malignant melanoma, 14 investigated, treated and followed at the Ophthalmology Clinic of the County Emergency Clinical Hospital of Tîrgu Mureş, and 7 at the "Dr. Constantin Papilian" Military Emergency Hospital Cluj Napoca. The studied period was 2005–2009. In all the cases enucleation of the eye globe was carried out, followed by histological examination.

The main criterion for including a patient in the study was the presence of histologically confirmed choroid melanoma in the enucleated globe. Ciliary body melanomas, intraocular tumors of other etiology, one patient receiving conservative treatment, and another patient who refused the enucleation, were excluded from the study.

All cases were identified from clinical registries. The data collected were: general clinical data about the intraocular tumor, imaging examinations, the followed treatment and the results of the histological examination.

Clinical examination contained checking of visual acuity, slit lamp examination indirect ophthalmoscopy, intraocular pressure measurements. Every patient was examined by A/B ultrasound, computer tomography and/or nuclear magnetic resonance for a closer look on the tumor. These imaging examinations were essential when the transparency of the ocular media was diminished (cataract, corneal edema). The characteristics of the tumors were described and measurements were done at each examination.

The staining used for grading was the common Hematoxilyn-eozin (HE). Immunological staining were for CD31 – normal, mature vessels, CD105 – newly formatted vessels, Collagen IV – basement membrane, Smooth Muscle Actin (SMA) – for the smooth muscle tissue from the vessel wall. The stained sections were scanned by a digital scanner (Zeiss Mirax Scan) at the Department of Pathology of the County Emergency Clinical Hospital of Tîrgu Mureş. The digitalized data was visualized with the

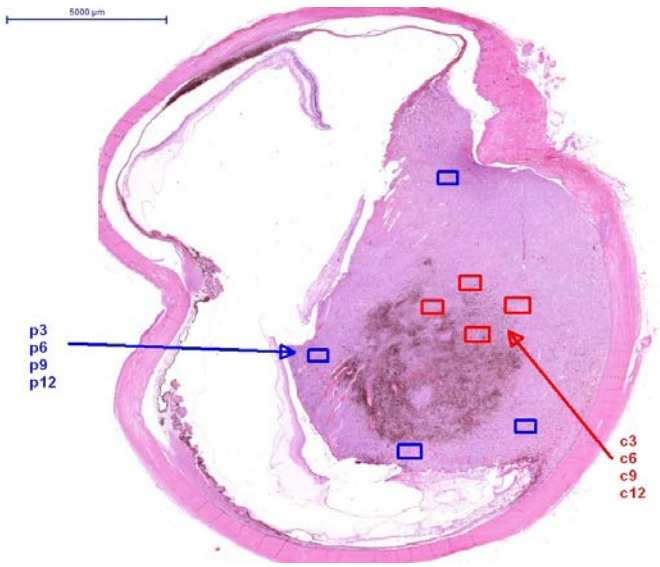

Fig. 1. Selection of "hot-spot" areas

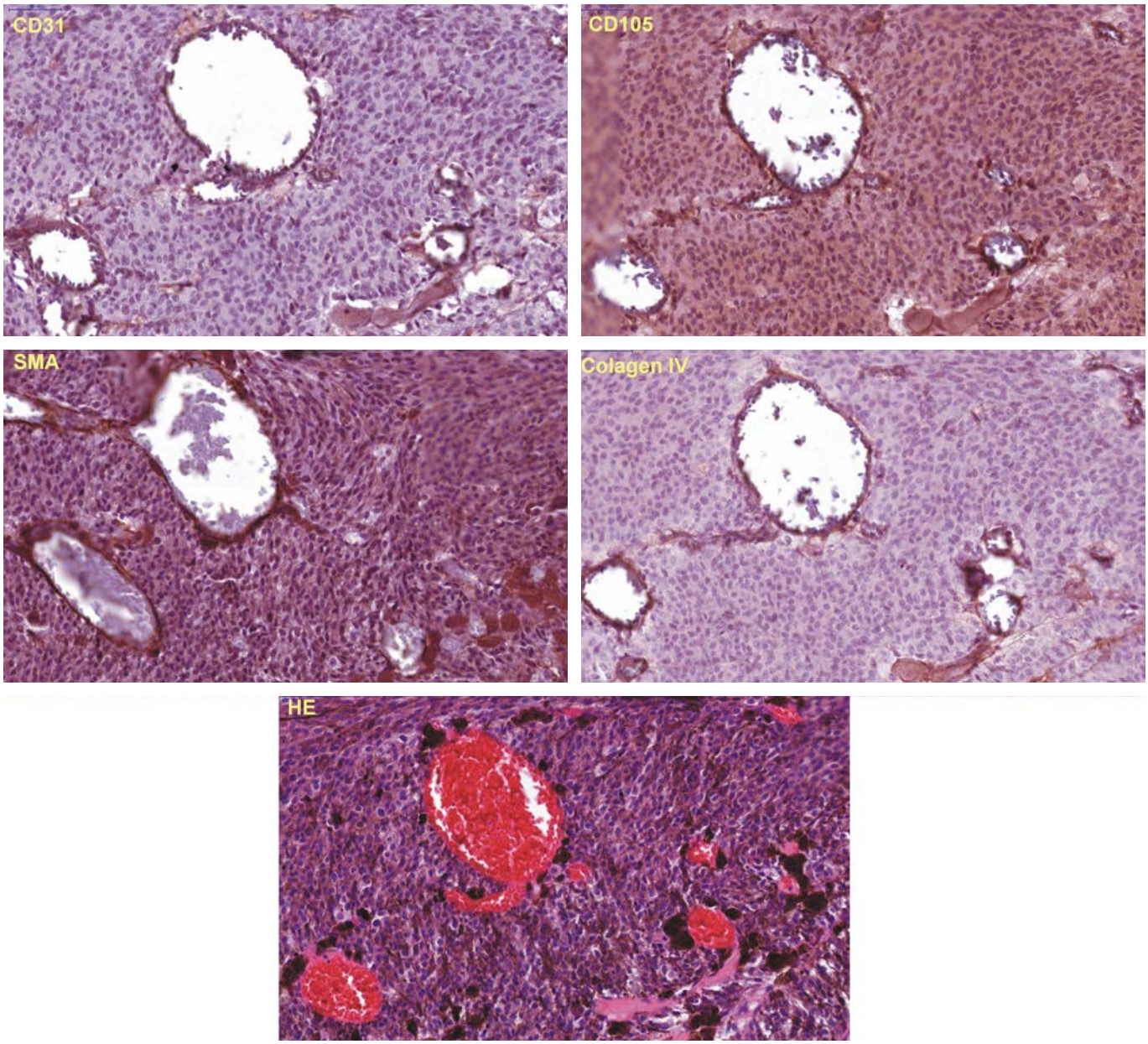

Fig. 2. Case S227423 – p6 – Superposable sections with different stainings, 40x

aid of the Pannoramic Viewer 1.15 RTM, 3DHistech program, and area selection was done. For every section 4 central and 4 peripheral areas were selected, a "hot spot" area at 40× magnification (1760×1050 pixels, representing an area of 660×430 μm) (Figure 1). These areas were digitally elaborated with the use of ImageJ 1.43m (Wayne Rasband, National Institute of Health, USA) software [2]. The selected areas for each section of a tumor can be superposed (Figure 2). In the digital processing all the images were normalized by special filters for each staining. Student's t test was used for statistic processing of the data.

Table I. Mean values of tumor dimensions

	MTD (mm)	BD (mm)
CT/NMR	16.52±2.97	11.87±3.86
Ultrasound	15.86±2.25	10.74±3.56
Morphometry	15.4±3.04	13.75±3.15

Table II. Melanoma incidence after CMOS (Collaborative Ocular Melanoma Study) criteria

<10 mm	11–15 mm	>15 mm
2	9	10

Results

The cohort examined was made up of 8 women and 13 men, with ages between 44 and 84 years, with a greater incidence of 7 cases in the 60–70 years age group. The shape of the tumor was dome-like in 7 cases, mushroom-like in 11 cases, and filled the eye entirely in 3 cases. Maximal tumor diameter (MTD) and basal diameter (BD) were evaluated by ultrasound, computer tomography (CT)/nuclear magnetic resonance (NMR) and morphometrically by the pathologist (Table I).

The average dimensions of the tumors differ by the method used, but without any statistically significant dif-

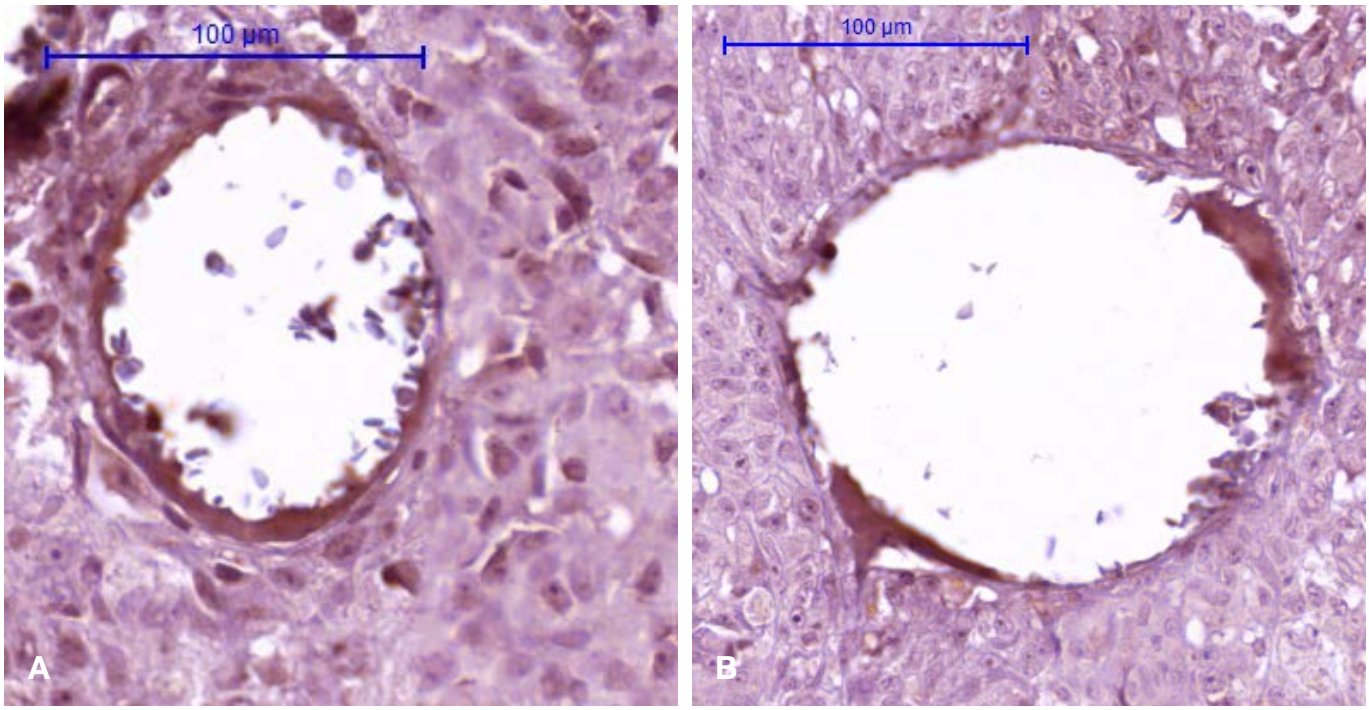

Fig. 3. CD31 reaction, 40x. A. normal vessel, B. basal vessel

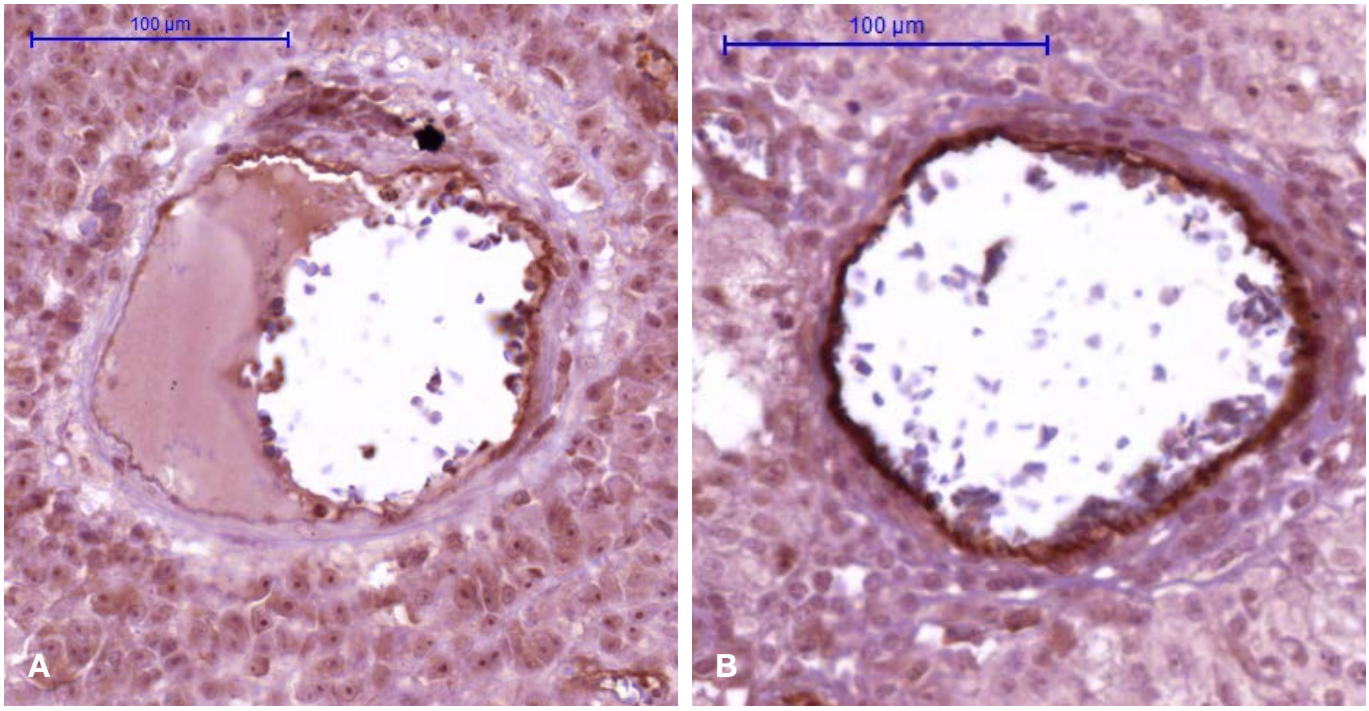

Fig. 4. CD105 reaction, 40x. A. normal vessel, B. basal vessel

ference. We classified the tumors after their dimensions, according to the Collaborative Ocular Melanoma Study (COMS) in three groups [3] (Table II.)

According to Table II, the majority of cases were in the third group, meaning big tumors, which are neglected cases, the patients presented at a specialist only when complications occurred.

The cellular composition of the tumors was of one type only in four cases, the others had a mixture of spindle cell or spindle and epithelioid cells.

The morphometric study was based on comparing the immune-positive surfaces (endothelial area for CD31 and CD105), after measurements have been made with the ImageJ software on the selected areas. Every formation that had shown positivity to the stain in question has been taken into account. This means that every cell, or group of cells with CD31 and/or CD105 positivity, regardless of its lumen, was considered a vascular structure.

The immunochemical staining of CD31 positive cells marks a mature, differentiated endothelial lining that is

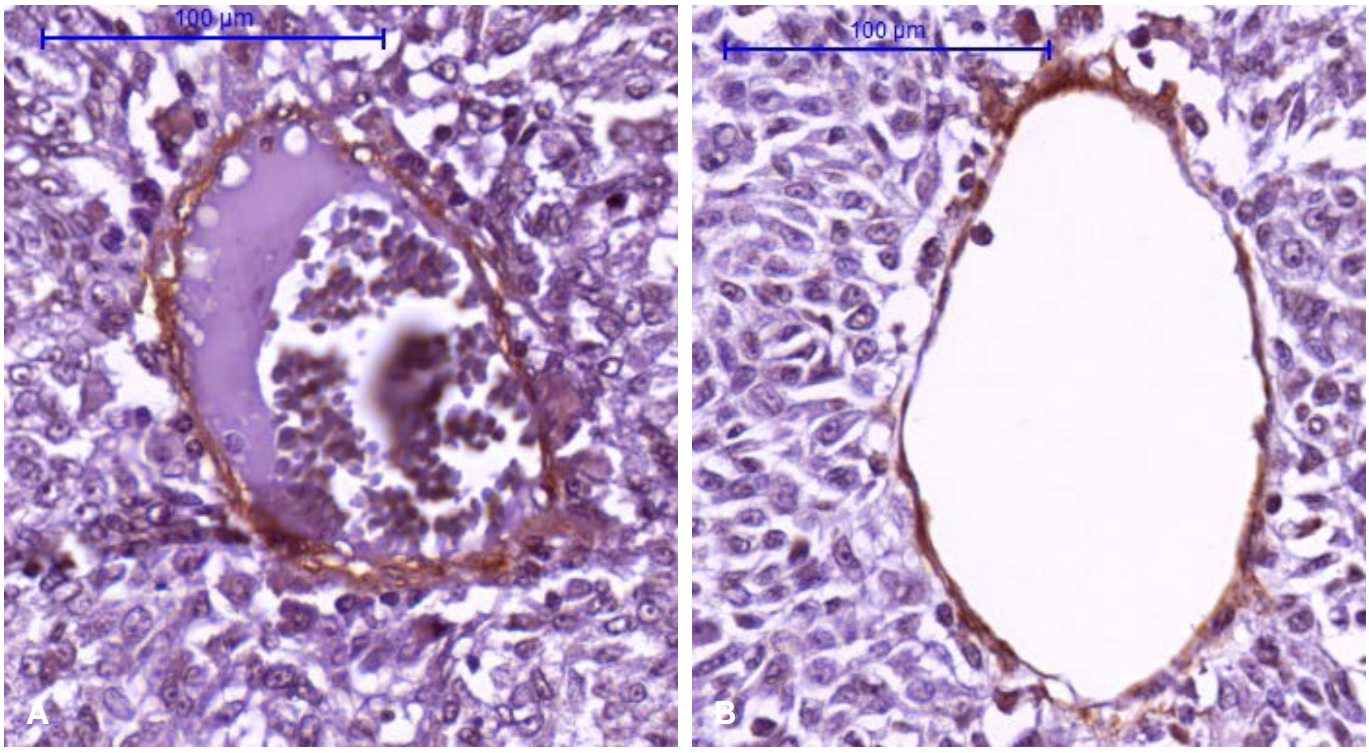

Fig. 5. SMA reaction, 40x. A. normal artery, B. basal vessel

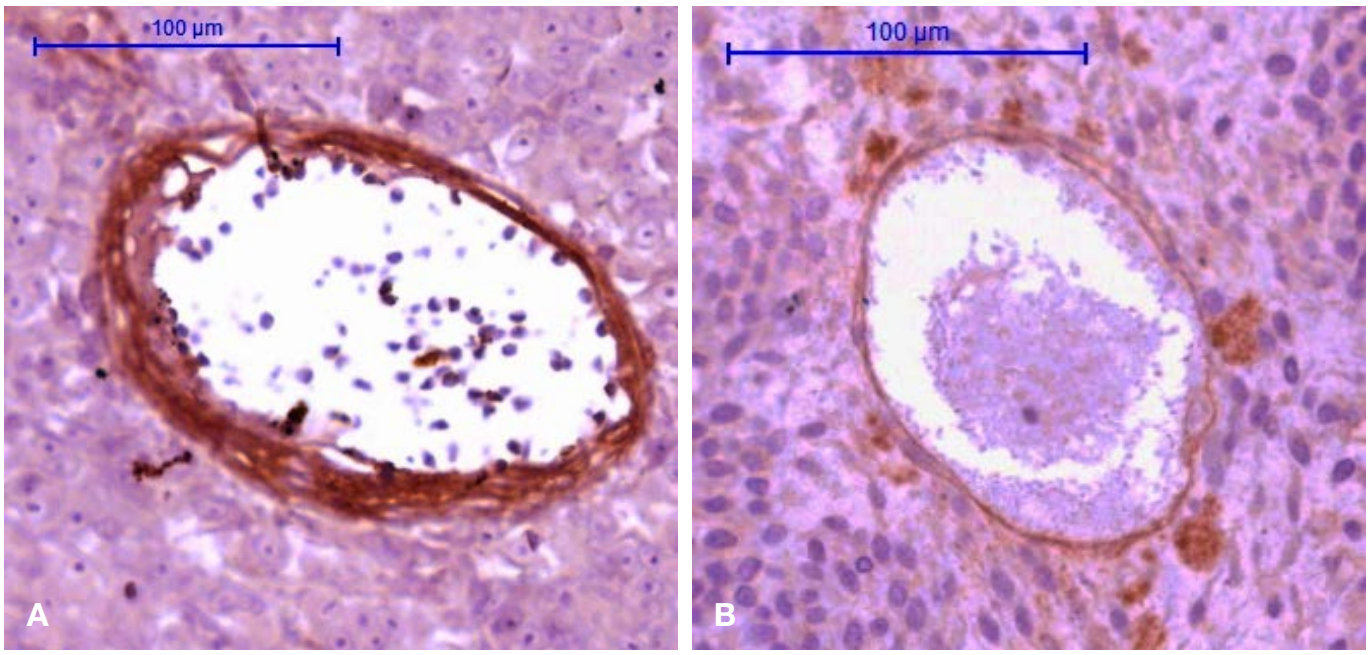

Fig. 6. Collagen IV reaction, 40x. A. central vessel, B. basal vessel

shown in the majority of peripheral tumor vessels. In central areas the staining gets stratified because of hyperplasia, and in some cases these even disappear (Figure 3). After the digitized processing of the images we calculated mean immune-positive surfaces (MIPS), obtained peripheral and central values with a statistically significant difference, p=0.0017 (central 1.2%, peripheral 2%).

The newly formatted, immature tumor vessels were stained with CD105. Tumor vessels that showed CD105 positivity had a fragmented, discontinues endothelial wall, with an increased percentage of migrated cells in the wall structure or in the vessel surrounding tissue (Figure 4). The morphometric evaluation of MIPS of CD105 positivity showed increased presence in central areas 2.93% vs 1.88% in the periphery (p=0.0054). With the help of Student's t test, on central areas stained with CD31 and CD105 we evidenced a statistically significant difference (p<0.001) in the favor of CD31 positivity. This difference cannot be found at the periphery, where both stainings have almost equal positivity (p=0.6561).

Morphometric devices applied on vessels with smooth muscular wall (muscular type), SMA staining showed

structural modifications, thinning, splitting and disappearing of the continual median smooth muscle (Figure 5). These modifications could be followed in central areas and partially at the periphery, without an obvious difference (2.61% vs 2.95%, p=0.3930).

The basement membranes were visualized by the marking of the type IV collagen fibers. Microscopic evidence was found that the fibers presented ruptures, splitting, brunching and thickening of vascular basement membranes with intrusions deeper in perivascular and intercellular space (Figure 6). The immune-morphometry stated this modification being more obvious in central areas rather than peripheral areas, with a statistical significant difference (p<0.001) between MIPS of Collagen IV.

Discussions

The vasculature and tumor growth of malignant melanoma of the uveal tract, especially the choroid, has some particularities [4]. Vascular endothelial cells can be identified by immunohistochemical staining, which gives the opportunity to objectively quantify the angiogenesis. The markers of these cells can be constitutional, present on every healthy and pathologic cell, and can be induced by pathologic conditions. Because the number of markers is high, we examined two of them to appreciate angiogenesis, one of them to evidence differentiated vessels and the other to show undifferentiated vessels with activated endothelium. Angiogenesis can be quantified in numerous ways, and so the results are rather different, and sometimes controversial in similar studies. The differences can occur from numerous causes, such as the differences between two sections of the same tumor, the vessels that are counted in the tumor, if the vessels are peri-tumoral or at the distance. Obviously, all these generate differences in the results [5].

Sallam and Hungerford stated that the vascular density is higher in larger tumors and in ones with epitheloid cells. The survival rate is also smaller in tumors with higher tumoral vascular density [6].

Pe'er et al consides that uveal melanomas can be classified in two categories regarding the survival rate, these being low-risk and high-risk. Dividing the prognostic factors into clinical and histological factors, he considers that the form of vasculature is the most important histological prognostic factor [7].

The structural modifications that we found sustain the affirmations of Folberg, who describes uveal melanoma tumor vessels to be coated with tightly placed tumor cells and not by mature endothelial cells. He calls it vasculogenic mimicry, this being a unique mechanism of aggressive uveal melanoma cells to express the phenotype of endothelial cells and form "vessel-like" three dimensional functional networks. The etiology of angiogenetic phenotype is still unclear in melanomas [8]. A possible explanation of vasculogenic mimicry is the appearance of aggressivity genes that are similar or identical with those of vasculogenesis (vasculo-endothelial caderin, laminine, erythropoetin). Vasculogenic mimicry was described in several tumors such as breast, prostatic, ovarian, pulmonary cancer, choriocarcinoma, synovial sarcomas, rhabdomiosarcomas, Ewing sarcoma and feocromocytoma [9]. These observations open new ways of treatment, that are investigated already on animal models [10].

Conclusions

The modification of wall structure, as well as the proportion of structural elements caused the severe lesion of the vascular wall. In the center of the tumor arteries and veins cannot be distinguished. They all became vascular structures with tumoral endothelium and irregularly thinned walls. The PAS and Collagen IV positive basement membranes have irregular thickness with variable stratification that is shown in CD31 positive vessels. Around the CD105 positive endothelium coated vascular gulfs the basement membrane disappears, its place being taken by dense tumor cells.

The CD31, CD105 and Collagen IV immune-positive areas have statistically significant differences, as it was proved by the dominance of CD105 (in comparison to CD31) in central areas.

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