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Benign Vascular Proliferations - an Immunohistochemical and Comparative Study

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Hemangiomas constitute a heterogeneous group of benign vascular proliferations. Their pathogenesis is not completely understood. In this study the expression of GLUT-1, VEGF, Ki-67, bcl-2 and apoptotic index in twenty six cases of infantile capillary hemangioma (ICH), fifteen of lobular capillary hemangioma (LCH) and nine of epithelioid hemangioma were examined. The expression of GLUT-1 was confined to ICH group and was completely absent in LCH and EH. The significant differences in the expression of VEGF, proliferative index and apoptosis between ICH, LCH, EH were found. The proliferative index and VEGF expression were highest in LCH. Apoptotic index was similar in LCH an ICH but negative in EH. The lesions examined did not differ significantly in bcl-2 expression. The GLUT-1 expression was not only Ki-67 but also apoptosis independent. Our findings might reflect the differences in biology and pathogenesis of hemangiomas.

Introduction

Benign vascular proliferations include a variety of different lesions, grouped under common but not precise designation "hemangioma". The pathogenesis of these lesions is not completely understood. Some "hemangiomas" are definitively considered neoplasms (infantile capillary hemangioma), some are thought to represent reactive inflammatory lesions (lobular capillary haemangioma, epithelioid hemangioma) and some - vascular malformations (arteriovenous hemangioma). New variants or entities are being described. Some of such new entities are: acquired elastotic hemangioma, microvenular hemangioma, and capillary nonprogressive hemangioma [1, 14, 19].

The aim of the study was to assess GLUT-1 expression in the infantile capillary hemangiomas, lobular capillary hemangiomas and epithelioid hemangiomas. GLUT-1 expression was compared with the expression of proliferation (Ki-67) and apoptosis markers (ApopTag and bcl-2) as well as VEGF expression. The relationship between the factors included in the study was assessed both in the whole material and in each of diagnostic categories.

Materials and Methods

The material consisted of 26 infantile capillary hemangiomas (ICH), 15 lobular capillary hemangiomas (LCH) and 9 epithelioid hemangiomas (EH) of the skin. Three cases of cavernous hemangiomas served as controls. The ICH, LCH and cavernous hemangioma cases were obtained from the files of the Department of Pathology of Jagiellonian University Children's Hospital. The EH cases were obtained from the files of the Department of Pathomorphology of Jagiellonian University Collegium Medicum. All the material has been fixed in formalin, routinely processed, and paraffin embedded. From the paraffin blocks 4μm-sections were prepared. For each case sections stained with hematoxylin-eosin and unstained sections for immunohistochemistry were prepared.

The primary antibodies used for immunohistochemistry are listed in the Table 1. The staining was done by standard method. Briefly, the slides were rehydrated and incubated in 3% peroxide solution for 10 minutes to block endogenous peroxidase activity. Antigen retrieval was carried out by microwaving in citrate buffer (0.2% citric acid titrated to pH 6.0 with 2N NaOH) 3x5 minutes at 750W. The ENVISION+ and ENVISION (DAKO, Denmark) detection systems were used. 3-amino-9-ethylcarbasole (DAKO, Denmark) was used as the chromogen. The slides were contrastained with Mayer hematoxylin (DAKO, Denmark). The processing was done using the DAKO Autostainer device (DAKO, Denmark).

Apoptotic cells were detected using ApopTag Peroxidase Kit (S7100, Intergen Company), *in situ* labeling the free 3'-OH DNA termini. The staining was performed according to the manufacturers' protocols.

The results of staining were evaluated and scored by two of the authors (GD and KO) by consensus, using a Nikon Labophot-2 multihead microscope, equipped with a 40x lens. All scoring was done in a blinded manner, i.e. without knowledge of the diagnosis or clinical data. The staining of endothelial cells only was taken into account. The endothe-

TABLE 1
The antibodies used for the study

specificity	antibody type	dilution	source
GLUT1	MYM rabbit policlonal	1:50	DAKO
bcl-2	clone 100	stock	Immunotech
Ki-67	clone MIB-1	stock	Immunotech
VEGF	clone C-1	1:100	Santa Cruz

lial cells were defined as cells lining definite vascular lumina or solid areas where CD34 and vWf labeling was positive.

For Ki-67 and ApopTag nuclei with strong granular brownish staining were assumed to be positive. Reaction for bcl-2 was assumed positive when the whole cytoplasm of the cell was stained. The Ki-67 positive cells were counted in 10 fields of vision with 40x lens (0.159mm^2) or in whole section if smaller. The average value in each case was calculated for 10 HPF. The staining for GLUT-1, bcl-2, apoptosis and VEGF was scored semiquantitively. The proportion of cells was scored from + to +++ as:

+ <10 % of positive cells
++ 10 - 50% of positive cells
+++ >50% of positive cells.

The intensity of the GLUT-1 staining was compared to the erythrocytes present in the vascular lumina (internal positive control) and was scored weak, when it was weaker, or intense when equal or stronger.

The statistical analysis was carried out using the Statistica v.5.5A PL software (StatSoft Inc. Tulsa, OK, USA). U Mann-Whitney, Kruskall-Wallis ANOVA tests, Spearman's and gamma correlation coefficients were used, when appropriate. The significance level was set to p=0.05.

Results

The study group was composed of 53 cases. In infantile hemangioma group mean age was 4.5 years, range: 4 months to 15 years; in lobular capillary hemangioma group mean age was 9 years, range: 8 to 15 years; in epithelioid hemangioma group mean age was 32 years, range: 6 to 55 years.

The results of the immunohistochemistry and Apop Tag labeling are given on Figures 1 and 2. Statistically significant differences between the three categories under study are seen in all marker but bcl-2 (Table 2). The median of VEGF expression was (+) in whole material, but (-) in the ICH group, (++) in the LCH group, and (-) in the EH group. The difference was statistically significant (Kruskall-Wallis ANOVA H=16.04, p=0.0003). The median of GLUT-1 expression was (-) in all groups, but (+) in the ICH group, (-)

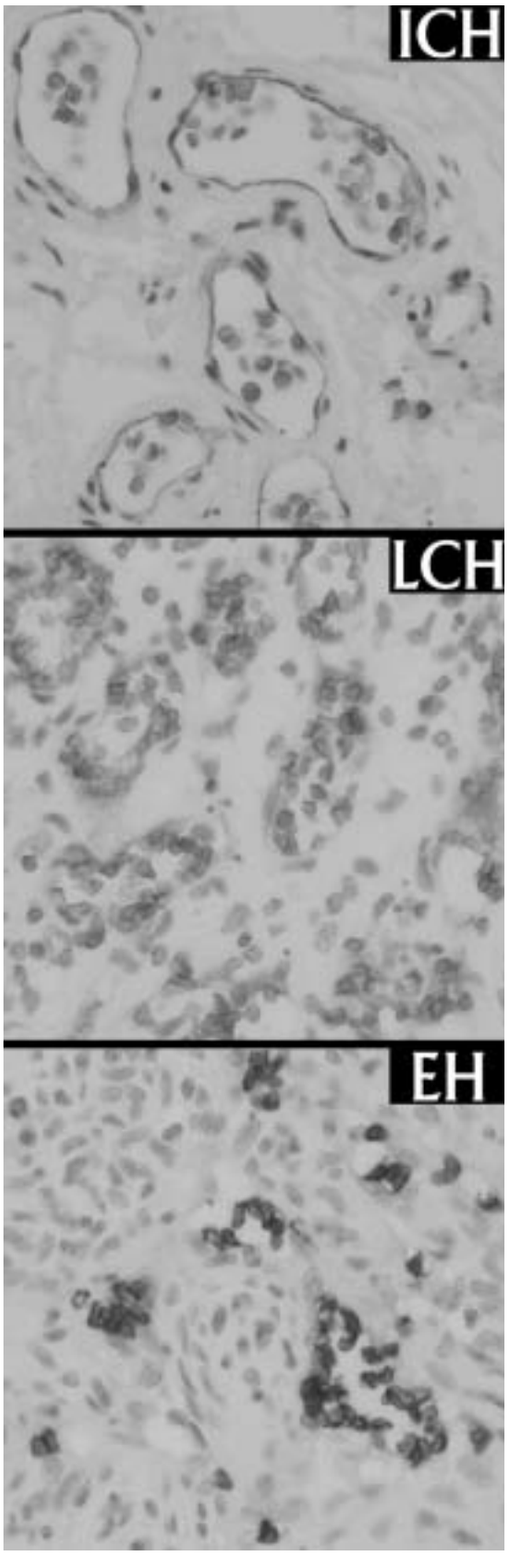

Fig. 1. GLUT-1 protein expression in hemangiomas. **Top**: in infantile capillary hemangioma the endothelial cells show membranous reaction, stronger than erythrocytes in vascular lumina. **Middle & bottom**: in lobular capillary hemangioma and epithelioid hemangioma no endothelial reactivity is seen, whereas luminal erythrocytes are positive (internal control). Lens magn. 60x.

TABLE 2

The significance levels of differences in the expression of markers studied between the three groups studied (Kruskall-Wallis ANOVA) and between each pair of groups (Mann Whitney U test). Non-significant (>0.05) values are omitted

	Kruskall-Wallis ANOVA	Mann Whitney U test		
		ICH vs. LCH	ICH vs. EH	LCH vs. EH
VEGF	0.0004	0.0008		0.0021
Ki-67	0.0021	0.0007		0.0458
GLUT-1	0.0001	0.0004	0.0028	
ApopTag	0.043		0.0248	

in the LCH group, and (-) in the EH group. The difference was statistically significant (Kruskal-Wallis ANOVA H=23.23, p<0.0001). The mean of Ki-67 expression was 7.96 (SD 10.10) in all groups, but 4.85 (SD 9.74) in the ICH group, 14.13 (SD 10.05) in the LCH group and 5.98 (SD 6.68) in the EH group. The difference was statistically significant (Kruskall-Wallis ANOVA H=12.46, p=0.0020). The median of bcl-2 expression was (-) in all groups, and the same in ICH, LCH and EH. No statistically significant difference was seen (Kruskall-Wallis ANOVA H=2.99, p=0.22). The median of ApopTag labeling index was (++), and the same in ICH and LCH; in EH group median ApopTag was (-).The difference was statistically significant (Kruskall-Wallis ANOVA H=6.25, p=0.04).

Significant correlations were found between VEGF and Ki-67 (gamma 0.39, p<0.001), Ki-67 and GLUT-1 (gamma -0.37, p<0.009), GLUT-1 and ApopTag (gamma 0.38, p<0.02), GLUT-1 and VEGF (gamma -0.36, p<0.03).

Discussion

Capillary hemangiomas are the most common tumors of infancy. Their prevalence is approximately 1 - 3% of all neonates and about 10% by the end of the first year of life [20]. Usually they present a predictable, self-limiting behavior: appear after birth, tend to grow up, but after their growth is stopped they involute spontaneously. In each phase of development they express different immunohistochemical markers [23].

In the first phase hemangiomas reveal high proliferative rate and overexpress angiogenic factors, as VEGF family members (bFGF, VEGF). Vascular endothelial growth factor present in the cytoplasm of pericytes and endothelium acts as a mitogen and it is potent in stimulating the proliferation of endothelial cells in auto- or paracrine way [5, 6]. Other vascular lesions or nonvascular neoplasms can also express VEGF, reflecting their proliferative activity. Thus the presence of this marker is not useful in differentiation of vascular lesions [5].

In our study VEGF expression in infantile hemangioma group was lower than in lobular capillary hemangioma group. This can be explained by the fact, that the majority of ICH cases were excised in involuting or involuted phase. No correlation was found between the expression of VEGF and other markers examined. However, the alteration of VEGF pathway may play an important role in the clonal expansion of endothelial cells, which results in hemangioma formation. The acquired, somatic mutations of VEGF receptors, having tyrosine kinase activity, were described in two cases of juvenile hemangioma. Those mutations in VEGFR-2 (Flk-1) and VEGFR-3 (Flt-4) seem to promote endothelial proliferation by gain of function of those receptors, which appear to act as pro-angiogenic factors [22]. Moreover, the up-regulation of VEGFR-1 and VEGFR-2 was found in few cases of other vascular neoplasms, like hemangioblastoma and hemangiopericytoma [9].

In the proliferative phase capillary hemangiomas express also bcl-2. This protein acts as an inhibitor of apoptosis. It was noted that bcl-2 up-regulation subsides during the infantile hemangioma evolution and its level declines during involution phase. It can suggest bcl-2 involvement in both proliferation and programmed cell death in the hemangioma evolution [7, 11].

Apoptosis seems to play a role in the spontaneous involution of hemangiomas. The frequency of apoptosis in ICH, as demonstrated in this and our previous study as well as reported by other authors, is much higher than in LCH or EH, albeit the absolute incidence of apoptosis is rather low [7, 10].

The mechanisms regulating the apoptosis in infantile capillary hemangiomas are unknown. There are reports showing that clusterin/apolipoproteinJ, a multifunctional glycoprotein associated with apoptosis is expressed in ICH when tumors progress from the proliferative to the involuting and involuted phase. Clusterin could be responsible for triggering apoptosis in infantile hemangiomas [17].

The therapeutic effect of interferon-alfa treatment on hemangiomas is thought to be related to induction of endothelial cell apoptosis [10, 21]. It has been shown also that accelerated involution of proliferating hemangioma caused by steroids administration is associated with up-regulation of mitochondrial cytochrome b gene expression. The expression of mRNA transcripts for cytochrome b in involuting phase of hemangioma tissue might support hypothesis that increased cytochrome b is related to apoptosis during regression [18].

GLUT-1, the erythrocyte type facilitative glucose transporter protein, is a member of a family of at least five proteins, each with a characteristic tissue distribution [8]. In normal tissue, GLUT-1 expression is restricted to endothelia at sites of blood-tissue barrier, including the brain, eye, nerve and placenta. It has been also observed in malignant neo-

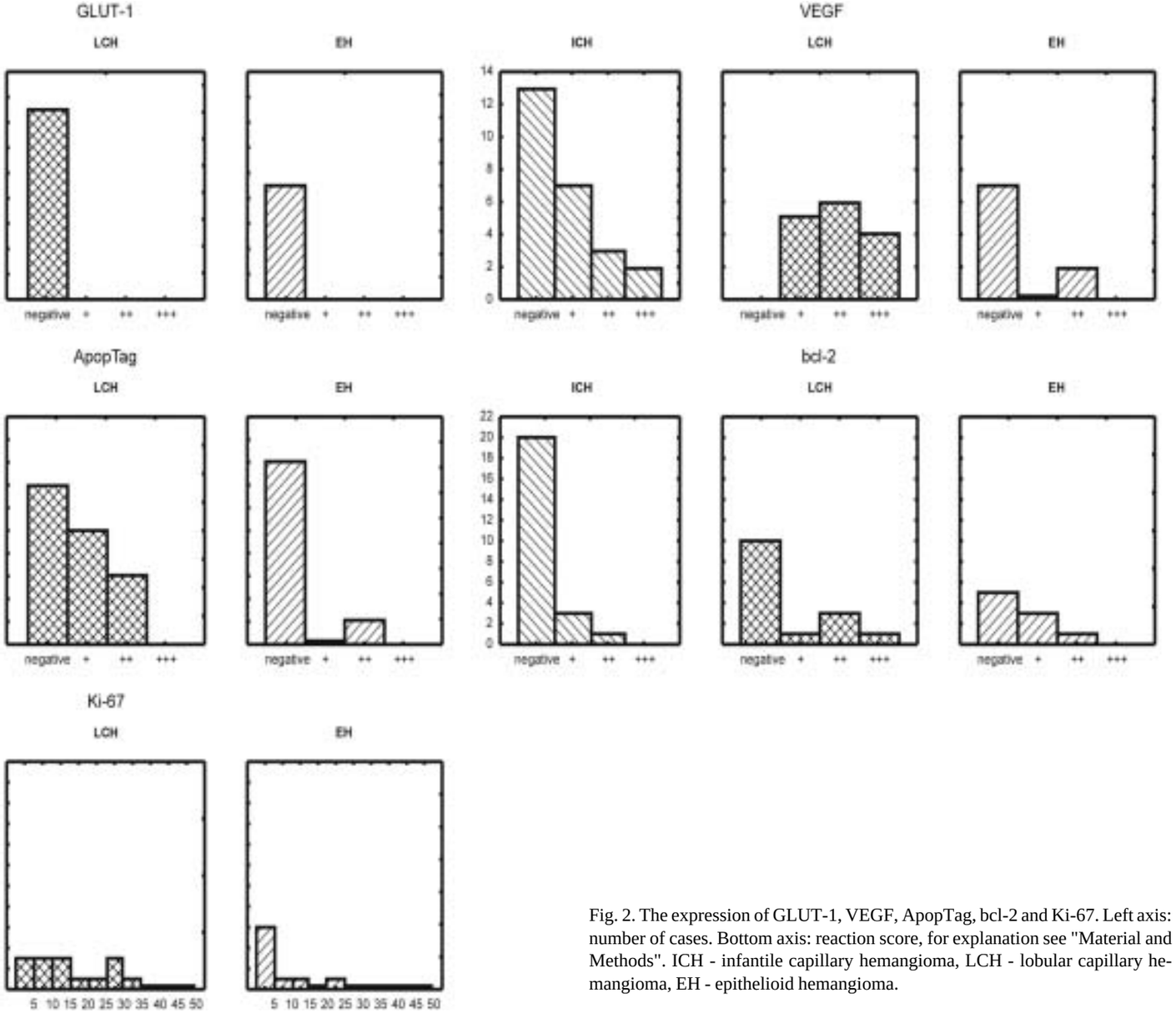

Fig. 2. The expression of GLUT-1, VEGF, ApopTag, bcl-2 and Ki-67. Left axis: number of cases. Bottom axis: reaction score, for explanation see "Material and Methods". ICH - infantile capillary hemangioma, LCH - lobular capillary hemangioma, EH - epithelioid hemangioma.

plasms and cerebellar hemangioblastomas [16]. In ICH, GLUT-1 is expressed at all stages of their evolution and represents an intrinsic feature of the committed endothelial phenotype of hemangiomas [15, 16].

In this study GLUT-1 expression was present only in ICH group, in 17 out of 26 cases. Its expression was more intense within the lesions with well developed vascular lumina. Our study confirmed that among lesions examined, GLUT-1 is a highly specific marker of infantile capillary hemangiomas. The use of this marker allows study of ICH as a singular entity. Though GLUT-1 is highly specific for ICH, lack of staining within vascular lesions does not exclude the diagnosis of this variant of hemangioma.

GLUT-1 seems to be independent of proliferative rate [16]. We have found inverse correlation between Ki-67 and GLUT-1 staining, though this correlation was not statistically significant. However, we have not noticed a correlation between GLUT-1 and apoptosis. What has not been, to our knowledge, reported to this time.

The presence of GLUT-1 within tissue with blood-barrier function suggests a possible association between infantile hemangiomas and these tissues. Immunohistochemical analysis of placental vessels and infantile hemangiomas revealed an unique coexpression of tissue specific markers, which implies a relationship between ICH and placenta and led to the new hypothesis on hemangioma formation. According to this, two pathogenic mechanisms would be involved. The first suggests an origin of infantile hemangiomas from invading angioblasts that aberrantly differentiate toward the placental microvascular phenotype in the mesenchyme of the skin and subcutis. The second mechanism suggests that hemangioma would originate from embolized placental cells, shedded intravascularly by placental injury and reaching fetal tissues from chorionic villi through right-to-left shunts [15]. On the other hand the evidence of clonal expansion of endothelial cells within ICH and somatic loss of heterozygosity on 5q in hemangiomas may imply that sporadic infantile capillary hemangioma is indeed neoplastic and caused by these mutations [2, 3, 22]. The presented various mechanisms may indeed represent independent pathways contributing to hemangioma formation or may suggest that several independent mechanisms are necessary for endothelial cell proliferation in hemangioma [22].

One of our cases shared some histological features with a newly described type of a GLUT-1 negative variant of hemangioma, called congenital nonprogressive hemangioma. Those lesions grouped primarily in the non-specific category of capillary hemangiomas are composed of multiple, well-defined lobules of proliferating capillaries, separated by bands of abnormal dense fibrous tissue. They are fully formed at birth and do not enlarge postnatally like infantile hemangiomas. Therefore they are unlikely to be pathogenically related to infantile hemangiomas [14].

Lobular capillary hemangioma is a common acquired lesion consisting in vascular proliferation in the skin and mucosa. Histologically, the vessels are lobularly arranged, with central feeding vessel and lobules of capillaries branching off from it. The lesion is well circumscribed. An inflammatory infiltrate is often visible within; therefore the often used misnomer "pyogenic granuloma" [13, 20]. Lobular capillary hemangioma is an entity distinct from infantile hemangioma both clinically and immunohistochemically. It grows rather rapidly and does not regress spontaneously. Expression of the markers of proliferation and apoptosis within LCH is in concert with its clinical behaviour. Indeed lobular capillary hemangiomas reveal lower apoptotic rate than infantile capillary hemangiomas [10] and their proliferation index is similar to that of ICH. In our study proliferation rate was highest in LCH group as was also VEGF expression. Strong VEGF signal noted in this and other studies suggests that vascular endothelial growth factor may be involved in the pathogenesis of pyogenic granulomas. In our study the expression of VEGF was equally intense within solid and more vascular areas. In the other reports marked VEGF positivity was noted mainly within vessels with less-developed lumina, composed of endothelial cell precursors or immature endothelial cells. That finding might suggest that VEGF acts as the autocrine agent stimulating endothelial growth [4]. VEGF gene expression was induced *in vitro* in endothelial cells by hypoxia or oxidative stress. Locally increased concentrations of reactive oxygen species strongly stimulate VEGF production by endothelium. Therefore, the existence of local inflammatory cytokine production might explain the induction of VEGF, and finally LCH formation.

Epithelioid hemangiomas (angiolymphoid hyperplasia with eosinophilia) are infrequent. A typical EH consists of a small nodule; is usually located intradermally or subcutaneously at the head and neck region or within mucosa. Extracutaneous lesions are rare and described locations include skeletal muscle, bone and salivary glands. The distinctive morphological feature of those tumours is the presence of small-sized vascular structures lined with distinct endothelial cells with epithelioid or histiocytoid appearance, round or vesicular nuclei and often vacuolated cytoplasm. The vessels are surrounded by an inflammatory infiltrate composed of eosinophils, plasma cells, and lymphocytes [12, 13, 20].

In this study we have shown the lack of GLUT-1 expression within epithelioid hemangiomas which, to our knowledge has not been reported to this time. The VEGF expression and proliferative rate were also lower than in infantile capillary hemangiomas and lobular capillary hemangiomas. EH has been found to express bcl-2 and other antiapoptotic molecules. This fact and inverse correlation between Ki-67 and bcl-2 noted in our previous study may

contribute to the hypothesis that the growth of epihelioid hemangioma is promoted by apoptosis inhibition only [7].

Histogenetically epithelioid hemangioma is considered to be of endothelial origin. It was found that EH contains relatively small amount of vessels lacking periendothelial cells. As periendothelial cells have the capacity to inhibit endothelial cell growth, hence they presence of many vessels without periendothelial cells may help to explain the mechanism of vessel proliferation in epithelioid hemangiomas. Some authors believe that EH represents an unusual manifestation of hypersensitivity, while others propose that it is benign tumour responding to estrogens, because of the presence of estrogen receptors in the lesions [12]. Finally some authors suggest, that epithelioid hemangioma is a reactive vascular lesion due to deregulated expression of vascular factors, trauma or a vascular malformation.

Conclusion

We confirmed that GLUT-1 is highly specific for infantile capillary hemangiomas but is not present in epithelioid hemangiomas. In our material GLUT-1 is not only Ki-67 independent but also apoptosis independent. We confirmed the existence of differences in immunoprofile of vascular lesions examined; this might reflect the differences in biology and pathogenesis of vascular lesions.

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