Angiogenesis in congenital vascular malformations: a dynamic view on a static lesion
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Microvascular proliferation in congenital vascular malformations of skin and soft tissue

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Abstract

**Background:** Congenital vascular malformations are mass-forming lesions that usually progress slowly, but may become symptomatic because of episodes of sudden growth and pain, particularly those with a substantial component of arteriovenous shunting.

**Aim:** To systematically investigate the features of microvascular proliferation in a large series of surgically treated vascular malformations.

**Methods:** 107 resection specimens of clinically and histopathologically well-documented vascular malformations were screened for the presence and extent of microvascular proliferation, based on morphological parameters, microvessel density (MVD), mast cell density (MCD) and proliferative activity (Ki67 labeling index) of endothelial cells (ECs) and vascular smooth muscle cells (VSMCs). The extent of microvascular proliferation was correlated with the histopathological type of vascular malformation and clinical characteristics of patients.

**Results:** Microvascular proliferation was observed in 32 (30%) of all vascular malformations, of which 30 cases seemed to be arteriovenous malformations. The mean value (SD) of MVD in areas of microvascular proliferation was 282 (186)/mm² versus 13 (9)/mm² in areas with mature vessels. Both ECs and VSMCs in these areas showed high Ki67 labeling indexes (15 (18)/mm² and 17 (24)/mm², respectively). In all lesions, a positive correlation was found between MCD and MVD. Age, sex and location of vascular malformation had no predictive value for the occurrence of microvascular proliferation. However, if present, the involved tissue areas were larger and the proliferative activity of ECs was higher in male patients than in female patients.

**Conclusion:** Recognition of microvascular proliferation as a not uncommon feature in congenital arteriovenous malformations provide new insight into the growth behavior and vascular composition of these lesions.
Introduction

Vascular malformations are congenital anomalies that result from localized errors of angiogenic development during embryonic life.1 Vascular malformations may occur at any topographic site, but have a predilection for skin and soft tissue of the head and extremities.2 Familial occurrence has been reported and although most vascular malformations are solitary lesions, they may also occur in various types of dysmorphic syndromes.3,4 Vascular malformations tend to progress slowly, but in the long term (usually after many years) serious complications may occur, which then require extensive surgical excision or even amputation. Moreover, because of ill-defined borders, the rate of recurrence after excision is high.2 In children, malformations should be distinguished from infantile hemangiomas, which are the most common vascular tumors of infancy.5 Mulliken and Glowacki6 categorised vascular anomalies in either hemangiomas or malformations on the basis of difference in growth behavior, endothelial cell (EC) turnover and mast cell density (MCD). Although not being absolute, this classic dichotomy is still used in the present classification accepted by the International Society for the Study of Vascular Anomalies (ISSVA) because of its simplicity and clinical relevance.5,7,8 According to this classification, hemangiomas are lesions with microvascular proliferation in the initial phases of growth, whereas vascular malformations are stable lesions with mature vessels, which grow commensurately with the child. Despite this, few cases of florid proliferation of capillaries have been reported in the course of vascular malformations, and in our department we identified similar cases. In the present study, we systematically investigated the presence and extent of microvascular proliferation in a large consecutive series of resection and amputation specimens of vascular malformations. Furthermore, features of microvascular proliferation were correlated with the clinical characteristics of patients and the histological type of vascular malformations.

Methods

Study patients
In a retrospective cohort study, we reviewed the resection or amputation records of a consecutive series of patients with vascular malformations of soft tissue and skin who were treated between between 1984 and 2003 (n=179) in the Academic Medical Center, University of Amsterdam in Amsterdam, the Netherlands, which is a referral center for the management of vascular anomalies. Indication for surgical treatment consisted of pain or ulceration, and/or functional impairment and/or rapid growth (in
a period of weeks to months) complicating the vascular anomaly. Only resection or amputation specimens >3 cm, and of which one or more tissue blocks per centimeter of the malformation were available, were included for this study. On this basis, 109 cases were enrolled. Of all patients included, age, sex and topographic location of vascular malformations were recorded. The study was performed in accordance with the Declaration of Helsinki and the institutional medical ethics committee.

**Histopathology**
By using hematoxylin and eosin (H&E) and Elastic van Gieson (EvG) stained tissue sections, vascular malformations of all patients were classified as (1) venous malformation (VM): lesions composed of veins of variable sizes, often with thick vascular walls; (2) lymphatic malformation (LM): substantial component of dilated thin-walled lymphatic vessels of different sizes; (3) deep arteriovenous malformation (AVM): large numbers of tortuous arteries and/or reactive intimal changes in arteries and veins with fibrointimal thickening due to hemodynamic stress; and (4) superficial arteriovenous malformation/acral arteriovenous tumor (aAVT): localised nodular tumor of thick-walled arteries and veins of skin and subcutis. Tissue blocks of all vascular malformations were screened for the presence of microvascular proliferation, defined as “solid” areas of densely packed capillary vessels lined with plump endothelium. For evaluation of the extent of microvascular proliferation, a semiquantitative score was applied as follows: 0, absent; 1, single cluster of immature capillaries (<50 vessels); 2, multiple clusters; 3, extensive diffuse and solid proliferation, splitting up pre-existent tissue. Adjacent serial sections were mounted for additional immunostains.

**Glucose transporter type 1 reactivity of endothelium**
Glucose transporter type 1 (GLUT1) is a protein specifically expressed by ECs of infantile hemangiomas, both in the proliferative and in the mature phase of evolution of lesions. By contrast, the endothelium of vessels of vascular malformations always stains negative with this antibody. To exclude the possible cases of infantile hemangiomas in our series, we immunostained representative sections of each specimen with anti-GLUT1 antibody.

**Microvessel density**
For determination of microvessel density (MVD), sections immunostained with anti-vascular smooth muscle cell (VSMC) (smooth muscle actin-1; SMA-1) antibody were scanned at a low-power magnification (x40) to identify the areas of highest MVD (hot spots). In these areas, the number of vessels was counted at x200 magnification in 10 non-overlapping fields with the manual count option, Image
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Pro Plus. Similarly, MVD was calculated in 10 random areas of the same vascular malformation with only large mature vessels.

**Mast cell density**
The same areas, traced in adjacent sections immunostained with anti-tryptase antibody, were screened for the presence of mast cells. Numbers of mast cells were counted by an automated macro with the use of image analysis software, and expressed as cells/mm².

**Proliferative activity of vascular wall cells**
Proliferative activity of both ECs and VSMCs was assessed with anti-Ki67 antibody, recognizing a nuclear protein with a fundamental function in cell proliferation. For this purpose, sections were immuno doublestained with respectively Ki67/CD31 and Ki67/SMA-1. Proliferating Ki67-positive cells (ECs and VSMCs) were counted in 10 fields at x400 magnification both in hot spots and in non-proliferating areas. To gain insight into the diameter of vessels with a high proliferative activity, we calculated the number of Ki67 and ECs per mm EC lining (lumen circumference) and number of Ki67 and VSMCs per mm² media in relation to the vessel diameter of 150 vessels from 4 patients.

**Immunohistochemical methods**
Sections mounted for immunohistochemistry were deparaffinized and subjected to antigen retrieval in a microwave oven before staining. We used citrate buffer for GLUT1 and tryptase immunostaining and EDTA for the Ki67 immuno doublestaining. Endogenous peroxidase was blocked with 0.3% hydrogen peroxide in methanol for 20 min. For immunostaining with anti-mouse tryptase (dilution 1:1000, Chemicon, Temecula, California, USA), direct alkaline phosphatase visualization with Vector Red (Vector, Burlingame, California, USA) was applied. For the immunostaining with anti-GLUT1 (dilution 1:100, Dako, Glostrup, Denmark), a streptavidin–biotin complex/horseradish peroxidase method was used and antibody reactivity was visualized with diaminobenzidine tetrachloride. Ki67/CD31 and Ki67/SMA-1 immuno doublestaining was performed in a multistep technique using Ki67 antibody (clone SP6, undiluted, Labvision/Neomarkers, Fremont, California, USA), visualized by diaminobenzidine tetrachloride in a brown nuclear precipitate, followed by application of the second primary antibody (either anti-CD31, clone JC70, dilution 1:20, Dako; or anti-α1 actin, SMA-1, dilution 1:200, Dako) visualized by permanent red in a red cytoplasmic precipitate. Positive controls (sections of skin and infantile hemangiomas) and non-immune negative control sections were evaluated for each stain.
Image analysis
Digital images of immunostained sections were captured using a Roper Coolsnap CF digital camera and an RGB liquid crystal display filter (Cambridge Research Instrumentation, Woburn, Massachusetts, USA) mounted on an Olympus BX60 microscope. Image analysis was performed using Image Pro Plus 4.5 (Media Cybernetics, Silver Spring, Maryland, USA).

Statistical analysis
Binary logistic regression models were used to assess the relationship between clinical characteristics (sex, age and topographic location), histopathological type and the occurrence of microvascular proliferation. Normality was analyzed using the Kolmorov–Smirnov test. A paired t test was used for data with normal distribution (MVD, MCD and proliferative activity of EC), and Mann–Whitney U test when data were not normally distributed (proliferative activity of VSMC). For comparison of more than two variables (proliferative activity of EC/endothelial lining and proliferative activity of VSMC/median area), the Kruskall–Wallace one-way analysis of variance was used, followed by post hoc Mann–Whitney U test. The paired t test was used for differences in proliferative activity of ECs and VSMCs between men and women. Fischer’s exact test was used for differences in the extent of vascular proliferation between men and women. P-values <0.05 were considered significant. In all instances, statistical analysis was performed using SPSS V.11.5.

Results
Table 1 shows clinical characteristics and histopathological diagnosis of all cases. Of 109 vascular specimens, two showed strong GLUT1 immunoreactivity of endothelial lining of small mature vessels, which is highly specific for the mature phase of infantile hemangiomas. Accordingly, these lesions were excluded from the series of vascular malformations. The remaining 107 cases were classified as A VM (n=71), VM (n=21), LM (n=8) or aA VT (n=7). The areas of microvascular proliferation were found amidst the mature vessels of the malformation in 32 out of 107 (30%) cases. Most cases showed multiple small clusters (50%) or even diffuse infiltration of pre-existent tissues by immature vessels (38%; Figure 1), whereas only 12% of cases had a single cluster of microvascular proliferation in the resected material. Microvascular proliferation was observed in almost 42% of AVMs encountered in this series, and also almost exclusively in this type of malformation (30 of 32 positive cases were AVM). The other two positive cases were VM, which showed only a single small cluster of microvessels. In LM and aA VT, vascular proliferations were absent.
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Figure 1. A. Extensive (grade 3) solid microvascular proliferation amidst the large vessels of an arteriovenous malformation (H&E). B. Same area as in A showing distinct closely packed vascular structures in anti-1α actin immunostain (SMA-1). C. Details taken from boxed area in A showing solid appearing growth of swollen vascular cells with inconspicuous lumina (H&E). D. Details taken from boxed area showing well-outlined vascular walls of small vessels in SMA-1 immunostain. E. Details taken from boxed area showing the luminal endothelial component of the vascular proliferation in anti-CD31 immunostain.

Table 1. Clinical and histopathological characteristics of all patients with vascular malformations and the subgroup of patients with capillary angiogenesis in vascular malformations.

<table>
<thead>
<tr>
<th></th>
<th>All patients with vascular malformations</th>
<th>Patients with vascular malformations with vascular proliferation</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=107</td>
<td>n=32</td>
<td></td>
</tr>
<tr>
<td>Sex (M:F)</td>
<td>58:49 (1:2.1)</td>
<td>16:16 (1:1)</td>
<td>NS</td>
</tr>
<tr>
<td>Age (years)</td>
<td>23 (0-67)</td>
<td>25 (5-64)</td>
<td>NS</td>
</tr>
<tr>
<td>Localization, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Head and neck</td>
<td>49 (46%)</td>
<td>13 (41%)</td>
<td>NS</td>
</tr>
<tr>
<td>Trunk</td>
<td>17 (16%)</td>
<td>3 (9%)</td>
<td>NS</td>
</tr>
<tr>
<td>Upper extremities</td>
<td>17 (16%)</td>
<td>6 (19%)</td>
<td>NS</td>
</tr>
<tr>
<td>Lower extremities</td>
<td>24 (22%)</td>
<td>10 (31%)</td>
<td>NS</td>
</tr>
<tr>
<td>Histopathologic type, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AVM</td>
<td>71 (66%)</td>
<td>30 (94%)</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>VM</td>
<td>21 (20%)</td>
<td>2 (6%)</td>
<td>NS</td>
</tr>
<tr>
<td>LM</td>
<td>8 (7%)</td>
<td>0</td>
<td>NS</td>
</tr>
<tr>
<td>Acral AVT</td>
<td>7 (7%)</td>
<td>0</td>
<td>NS</td>
</tr>
</tbody>
</table>

Abbreviations: M, male; F, female; AVM, arteriovenous malformation; VM, venous malformation; LM, lymphatic malformation; acral AVT, acral arteriovenous tumor; NS, not significant.

There was no association between age, sex or localization and the presence of microvascular proliferation (Table 1). MVD in areas of microvascular proliferation was significantly higher (mean (SD) 282 (186)/mm2) than in adjacent areas of the
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malformation (13 (9)/mm²; paired t test p<0.05). Similarly, MCD was significantly higher in areas of microvascular proliferation (154 (97)/mm² in proliferating areas vs 35 (35)/mm² in areas with mature vessels; paired t test p<0.001; Figure 2A). Both ECs and VSMCs showed high Ki67 labeling indexes in proliferating areas (EC: 15 (18) vs 1 (2)/mm² and VSMC/pericytes: 17 (24) vs 1 (1)/mm²; Figure 2B). For this purpose, immune doublestaining allowed to discriminate clearly between Ki67 immunoreactivity of ECs and VSMCs in vessels (Figure 3). The correlation between Ki67 indexes of both cell types with vessel diameter showed proliferative activity predominantly in vessels <20 µm (representing the size of capillaries, arterioles and venules). Although sex was not predictive for the occurrence of vascular proliferation, we found that the Ki67 labeling indexes in both ECs and VSMCs were higher in proliferative areas of male patients than in female patients (Figure 4A). Moreover, the extent of microvascular proliferation throughout the lesion appeared also more prominent in male patients than in female patients (Figure 4B).

Figure 2. A. Microvessel density and mast cell density in proliferative (open bars) and mature (black bars) areas of vascular malformations of 32 patients with capillary angiogenesis. B. Ki67 labeling indexes of endothelial cells and vascular smooth muscle cells in proliferative (open bars) and mature (black bars) areas of the same patient group.

* p<0.001, ** p<0.05.

Figure 3. Details of immuno doublestaining to visualize Ki67 labeling in vascular smooth muscle cells and endothelial cells (ECs) respectively. A. Immuno doublestain with Ki67 (brown) and smooth muscle actin-1 (SMA-1; red), showing Ki67 positive nuclei of smooth muscle cells (SMCs) within the immunostained area (long arrow) and Ki67-positive nuclei of ECs on the outside of the immunostained area (short arrows; Ki67/SMA-1). B. Immuno doublestain with Ki67 (brown) and CD31 (red), showing several Ki67 positive nuclei of ECs within the immunostained vessel area (Ki67/CD31).
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Discussion

According to current insights into the biology of benign vascular lesions, congenital vascular malformations represent slowly progressive growing vascular masses composed of dysplastic but mature vessels. Despite this view, we identified areas of microvascular proliferation interpreted as angiogenesis, in 30% of cases of a large series of 107 surgically removed vascular malformations. These areas of microvascular proliferation contained closely packed small vessels (<20 µm) with a high Ki67 labeling index of both ECs and VSMCs. In most cases, those areas were multicentric (50%) or displayed a solid growth pattern, splitting up pre-existent adipose tissue and skeletal muscle (38%). Pain, swelling, rapid growth (in a period of weeks to months) and/or functional impairment were symptoms most frequently encountered in this population of patients, and these symptoms served as indicators for surgical intervention. It could be therefore that microvascular proliferation is involved in causing symptoms due to the mass-forming effect (Figure 1A, B). However, our data files did not allow correlating reliably specific symptoms of each patient with corresponding histopathological findings in all cases; moreover, other (well-known) tissue complications such as thrombosis or hemorrhage may also be encountered. Therefore, a potential role of microvascular proliferation in the onset of symptoms of patients remains purely speculative.

In our patient population, sex distribution was equal, and neither age or sex nor topographic location of malformations had a predictive value for the occurrence of foci of microvascular proliferations. Only the histopathological type of vascular malformations correlated significantly with the occurrence of microvascular...
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proliferation, since of all positive cases >90% were AVMs. This observation suggests that a high flow through arteriovenous communications, which is a hallmark of AVMs, could play a part in microvascular proliferation. The exact mechanism, however, remains to be elucidated. Despite equal sex distribution among patients with microvascular proliferation, we found that AVMs of men showed a higher proliferation rate in both ECs and VSMCs than those occurring in women (Figure 4A). Moreover, the semiquantitative score used to evaluate the extent of microvascular proliferation in vascular malformations was significantly higher in male patients (Figure 4B). This implies that, once it occurs, microvascular proliferation appears more prominent in male than in female patients with AVMs. Gender differences in the growth of VSMCs have been described only in animal models and have, to our knowledge, not been reported in human pathology. These differences in proliferation rate probably relate to a shorter cell cycle in male VSMCs than in those from female VSMCs. This could be because of effects of sex hormones, although, for example, oestrogens seem to accelerate rather than inhibit angiogenesis. Angiosarcoma has been reported as a rare complication of AVMs, and in cases with extensive solid proliferations a suspicion of malignancy (angiosarcoma) was raised. Despite this, malignancy was excluded based on detailed histopathological features, indicating benign capillary vessels, and clinical follow-up of patients.

The question whether microvascular proliferation in AVMs should be considered as a reactive phenomenon or alternatively as part of the congenital malformation is more realistic. Morphologically, microvascular proliferation in our study group was indiscernible from that occurring in proliferating lesions of infantile hemangioma, pyogenic granuloma and reactive angioendotheliomatosis. Takahashi et al have reported a high proliferative activity, defined by a high expression of proliferating cell nuclear antigens, in the growth phase of infantile hemangiomas, and almost no staining in the involuting stage of the same type of tumors. In this study proliferative activity was barely detectable in five vascular malformations included, which contradicts to our findings. This difference probably relates to sampling because of the small sample size and the fact that only one AVM was investigated. Mast cells, which are a rich source of growth factors that induce or modulate angiogenesis, were found in significantly increased numbers in areas of microvascular proliferation of AVMs. Beyond those areas, the mast cell counts were low in all vascular malformations. A similar correlation between areas of high MVD, morphological immaturity and high MCD has previously been reported also for infantile hemangiomas and reactive proliferations such as pyogenic granuloma. In the regressive stages of these lesions, when vessels mature or disappear, the amount of mast cells indeed decreases. The endothelial lining of capillaries in infantile hemangioma is characterized by strong immunoreactivity with GLUT1
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antibody, whereas in AVMs all vessels, including the immature capillary vessels we describe in our study, are GLUT1 negative. Pyogenic granuloma (which is GLUT1 negative), diffuse dermal angiomatosis (GLUT1 reactivity not reported) and acro-angiodermatitis (GLUT1 negative) are all considered reactive capillary lesions, and although their presence is limited to the dermal skin, such morphologic and immunophenotypic similarities could favor a reactive nature of microvascular proliferation in vascular malformations. Indeed, Bavikatty et al recently described florid vascular proliferations of the colon wall in five patients, of whom two had a possible AVM of the intestinal wall. These authors also believed this process to be of a reactive rather than a neoplastic nature, probably resulting from local tissue hypoxia, because of concomitant intussusception. One could speculate that capillary structures in vascular malformations undergo maturation by the time, as is also a well-known feature of the immature capillary vessels in infantile hemangiomas and pyogenic granulomas, and finally develop vessel areas indistinguishable from the areas of thin-walled small mature vessels that are present in most, if not all, AVMs of soft tissue. In summary, the present report provides new insights into the growth behavior and vascular composition of vascular malformations, particularly in those with an arteriovenous component.

Take-home messages
1. Florid microvascular proliferations are found in up to 30% of congenital vascular malformations, especially in those with a significant arteriovenous component.
2. Traditionally, vascular malformations are slowly growing lesions composed of mature vessels. By contrast, vasoproliferative growth is considered unique for infantile hemangiomas and reactive vascular proliferations. The outcome of this study is therefore important for differential diagnostic aspects of benign vascular tumours, and may provide new insights into the biology of vascular malformations.
3. Sex, age and topographic location of vascular malformation are not predictive for the onset of microvascular proliferation. However, once it occurs in a patient, the vasoproliferative growth seems to be significantly more extensive in men than in women.

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