Angiogenesis in congenital vascular malformations: a dynamic view on a static lesion
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Microvascular proliferations in arteriovenous malformations relate to high flow characteristics, inflammation and previous therapeutic embolization of the lesion

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Submitted
Abstract

**Background:** Episodes of microvascular proliferation associated with volume expansion are observed in arteriovenous malformations (AVMs) of skin and soft tissue.

**Objective:** To investigate the relationship between a microvascular proliferative response and flow velocity in AVMs.

**Methods:** Resection specimens of 80 AVMs were clinically categorized as either high or low flow lesions, and histopathologically screened for the presence of microvessels, inflammation and/or thrombosis. Immunohistochemistry was performed with endoglin (CD105), von Willebrand factor (vWF) and fibrinogen antibodies.

**Results:** Clinically, 37 AVMs were classified as high flow lesions and 43 as low flow. In 81% of high flow lesions microvascular proliferations were seen versus 14% of low flow lesions (p<0.005). In high flow lesions, which were embolized prior to surgery (30% of all), 88% showed microvascular proliferation, 88% inflammation and 33% thrombosis. However, similar vasoproliferative responses were also observed in non-embolized AVM (69% high flow and 14% low flow lesions). Endoglin was more frequently expressed in high flow lesions. Extracellular vWF staining was found in most lesions, irrespective of flow type or presence of microvascular proliferations.

**Conclusions:** Microvascular proliferative masses in AVMs appear to be strongly associated with high flow characteristics. This can be explained to some extent by tissue damage initiated by previous embolization and/or (related) inflammation, but the occurrence of the same microvascular responses also in AVM which were not embolized prior to surgery, also suggests a role for the biomechanical effects of high flow in this process.

**Capsule Summary**

1. Episodes of microvascular proliferation within congenital vascular malformations of skin and soft tissue may lead to symptomatic growth in a subpopulation of patients.
2. This study revealed that microvascular proliferative growth occurs significantly more often in clinically defined high flow vascular malformations than in low flow malformations.
3. Recognition of microvascular growth as one of the factors leading to symptomatic growth in high flow malformations could lead to targeted anti-angiogenic therapy in affected patients.
Introduction

Vascular malformations are hamartomatous masses of mature but malformed vessels, and are generally considered as congenital anomalies that are caused by errors in vascular development during embryonic life. Vascular malformations are sub-classified according to the predominant type of vessel of which the lesion is composed, that are capillaries, veins, lymph vessels, arteries or combinations of these vessel types. Clinically, it is customary to categorize vascular malformations according to the velocity of blood flow through the lesion as low (slow) flow or high (fast) flow lesions. Clinically determined high flow lesions are always arteriovenous malformations (AVMs) with a high vessel density due to localized arterial and venous hypertrophy. The lesions usually have several AV fistulas (direct communications between arteries and veins), whereas low flow lesions are the capillary, venous and lymphatic types of malformations or arteriovenous types with only limited AV shunting. AVMs were long considered to be static, quiescent lesions with a growth pattern typically in proportion to the growth of the child with a slow, steady enlargement because of collateralization, dilatation or thickening of vessels and without any tendency to regress. On the long-term, AVMs are often treated by means of embolization of the feeding vessels. However, it is now recognized that AVMs expand over time and may enlarge after treatment, which then requires resection of the large vascularized tissue masses or even amputation of an affected limb. In a previous study of 107 of such resection specimens of vascular malformations, we identified in 30% of all lesions the presence of masses of immature capillary vessels amidst the vessels of the malformation, often to such an extent that they were considered to have contributed to the disproportionate growth of the malformation and subsequent clinical symptomatology. Interestingly, the proliferations occurred more often in lesions that were histopathologically defined as arteriovenous types (42% of all AVMs), as opposed to purely venous types (VM), in which such proliferations were found in only 10% of cases. In lymphatic vascular malformations they were never observed. Since many arteriovenous malformations have fistulas (shunts) between arteries and veins, such an observational study suggests a relationship between high flow characteristics of the lesion and the presence of microvascular proliferative responses. This could fit with previous in vitro and experimental studies in animals showing that increased flow forces stimulate angiogenesis. In this study, we investigated in a series of clinically and histopathologically well documented cases of AVMs whether the presence of microvascular growth in resections of AVMs relates to clinical and/or radiological features of either high flow or low flow. To rule out other potential triggers of an angiogenic response in these tissues we also investigated presence of intralesional inflammation, thrombosis and previous therapeutic strategies, including embolization, in relation to angiogenesis in both types.
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of AVMs. Finally, we also investigated a possible relationship between an angiogenic response in high flow versus low flow types of AVMs and immunoexpression of markers for tissue hypoxia (endothelial expression of endoglin) and endothelial cell integrity (tissue expression of von Willebrand factor and fibrinogen) in both types of lesions.

Materials and Methods

Selection of study material of AVM

The study materials consisted of paraffin-embedded materials of 130 resection specimens of congenital vascular malformations, described earlier by us. Briefly, the patients were treated for symptomatic vascular malformations, and resection or amputation specimens of at least 3 cm which were adequately sampled for histopathological analysis were included. Of these, only the arteriovenous types of malformations were selected for the present study. An AVM was histopathologically defined as the presence of both tortuous arteries (several cross sections through arteries) and conglomerates of malformed veins in hematoxylin & eosin (H&E) and Elastic van Gieson (EvG) stained sections. This resulted in 82 cases of AVMs.

Clinical data of patients including flow characteristics of AVM

Retrospectively, the clinical data of these 82 cases were reviewed for information on flow characteristics of the malformation before surgery, particularly the presence of either high or low flow. Clinically, the flow in the malformations had been analyzed by physical examination (local warming, pulsatile mass with thrill-bruit), Doppler ultrasonography (high-flow low-resistance arteries, arterialized waveform in draining veins) and/or by using imaging techniques, including computed tomography (CT), angiogram and, in the more recent cases, also by using magnetic resonance imaging (MRI). Of two cases clinical data were inconclusive or not available for a proper diagnosis. On this basis a total of 80 cases was enrolled. Information on demographic data, topographic location of the malformation, flow analysis and prior treatments (embolization, surgery) of all patients are listed in Table 1.

Assessment of histopathological parameters

1. Microvascular areas

H&E sections were screened in combination with anti-CD31 (endothelial cells) and anti-SMA-1 (smooth muscle cells/pericytes) immunostained sections for determination of appearance of areas of microvascular proliferation in the lesions.
These areas were defined as solid appearing areas of closely packed capillary vessels lined with inconspicuous lumina and large swollen endothelial cells. For evaluating the extent of angiogenesis a semiquantitative score system was used as in a prior study: 0, absent; 1, single cluster of immature capillaries; 2, multiple clusters; 3, extensive diffuse and solid proliferation, splitting up pre-existent tissue.  
2. Inflammation  
The presence of inflammatory infiltrates within the malformation was scored in H&E stained sections of all lesions. In 11 randomly selected AVMs with substantial inflammation, the composition of the infiltrate was determined in immunostains with anti-CD3 (pan T-lymphocytes), HLA-DR (activated immune cells), anti-CD79a (B-lymphocytes), anti-CD68 (macrophages) and anti-trypatase (mast cells).  
3. Thrombosis  
Presence of thrombus was scored as fresh and/or organized blood clots within the outlines of the vessel walls of large vessels in H&E and EvG stained sections.  
4. Tissue markers for vascular leakage  
Von Willebrand factor (vWF) is stored in Weibel-Palade bodies of endothelial cells under normal conditions, and anti-vWF immunostains show reactivity in endothelial cells (ECs) only. However, in inflamed vessels and in atherosclerotic plaques, these immunostains show a distinct pericellular rim, which is considered as a marker for endothelial leakiness/damage. All specimens were scored for presence or absence of such a pattern of pericellular anti-vWF staining. Fibrinogen is plasma protein that also stimulates migration and proliferation of endothelial cells and anti-fibrinogen immunostains on normal tissues are invariably negative. In this study, extracellular anti-fibrinogen staining was interpreted as a marker for tissue damage.  
5. Endothelial expression of endoglin  
Endoglin (CD105) is a protein which is expressed on activated endothelial cells, especially under hypoxic conditions. Endothelium of quiescent vessels show only weak to absent staining, and distinct positive staining is considered as a marker of endothelial activation. In this study, >5% of CD105 immunopositive vessels was interpreted as positive. Resections of normal skin and subcutis (n=5) were used as reference material.  

**Immunohistochemistry**  
Paraffin tissue sections were dewaxed in xylene and rehydrated in graded alcohols, followed by blocking of endogenous peroxidase activity in 0.3% H2O2 in methanol for 20 min. Tissue pre-treatment was performed with heat-induced epitope retrieval (HIER) using either Tris-EDTA pH 9.0 (CD31, SMA, CD3, CD79a, HLA-DR, trypatase), citrate 6.0 (CD105, CD68), pepsin (vWF), or no pretreatment (fibrinogen). The following primary monoclonal antibodies were applied: CD31 (clone
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JC70, Dako, Glostrup, Denmark), SMA-1 (clone 1A4, Dako), vWF (clone F8/86, Dako), fibrinogen (Dako), CD105 (endoglin, Thermo Fisher Scientific, Fremont, CA), CD68 (clone PG-M1, Dako), tryptase (clone G3, Chemicon/Millipore, Temecula, CA) and a CD3/HLA-DR double staining consisting of CD3 (clone SP7, Thermo), HLA-DR (clone CR3/43, Dako), CD79a (clone JCB117, Dako). Single immunostaining was performed with diaminobenzidine tetrachloride (DAB) as a chromogen. For the CD3/HLA-DR double staining, HLA-DR reactivity was visualized in blue using Vector Blue and CD3 in red using Vector NovaRed (Vector Laboratories, Burlingame, CA). Positive control tissues consisted of human tonsils, normal skin and atherosclerotic plaque tissue (for vWF and CD105). Experimental tissue treated following the same procedures, but with omission of the primary antibodies served as negative controls.

Statistical analysis
Statistical analysis was performed using GraphPad software. A Chi square test was used to compare nominal variables. P-values <0.05 were considered statistically significant.

Results

Clinical data of patients including flow characteristics of AVMs
The flow in clinically apparent high flow lesions (based on physical examination) was in 89% of cases analyzed additionally with (combinations of) diagnostic imaging techniques. These imaging techniques were used in 63% of cases with low flow characteristics (Table 1). According to the characteristics of the lesions, 37 out of 80 (46%) could be classified as high flow, and 43 out of 80 (54%) as low flow AVMs. Sex distribution and median age of patients was similar in both groups. The majority of lesions were located in the head and neck area followed by the lower extremities.

Occurrence of microvascular proliferation in high versus low flow lesions
Following strict histomorphological and immunohistochemical criteria for presence of immature microvascular growth, we found a significant difference in occurrence of clusters or even diffuse sheet-like patterns of such vessels between high flow (30 out of 37 cases; 81%) and low flow lesions (6 out of 43 cases; 14%) (Table 2; Figure 1A and B). Semiquantitative scoring of the extent of microvascular growth revealed multiple clusters (score 2) in 13 cases (35%) and extensive diffuse and solid proliferation, splitting up pre-existent tissue (score 3) in 15 cases (41%) of the high flow types of lesions (Figure 2).
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Table 1. Clinical characteristics of all patients included in this study. High flow indicates patients with high/fast flow characteristics (n=37) and low flow indicates patients with low/slow flow characteristics (n=43) of their AVM.

<table>
<thead>
<tr>
<th></th>
<th>High flow (n= 37)</th>
<th>Low flow (n= 43)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (M:F)</td>
<td>17:20</td>
<td>21:22</td>
</tr>
<tr>
<td>Mean age (spread)</td>
<td>28 (5-64)</td>
<td>23 (5-67)</td>
</tr>
<tr>
<td>Median age</td>
<td>25</td>
<td>21</td>
</tr>
<tr>
<td>Localization</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Head and neck</td>
<td>16 (43%)</td>
<td>20 (46.5%)</td>
</tr>
<tr>
<td>Trunk</td>
<td>6 (16%)</td>
<td>3 (7%)</td>
</tr>
<tr>
<td>Upper extremities</td>
<td>5 (14%)</td>
<td>6 (14%)</td>
</tr>
<tr>
<td>Lower extremities</td>
<td>10 (27%)</td>
<td>14 (32.5%)</td>
</tr>
<tr>
<td>Flow analysis*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No imaging technique</td>
<td>4 (11%)</td>
<td>16 (37%)</td>
</tr>
<tr>
<td>Doppler ultrasound</td>
<td>2 (5%)</td>
<td>3 (7%)</td>
</tr>
<tr>
<td>X-ray</td>
<td>0</td>
<td>1 (2%)</td>
</tr>
<tr>
<td>CT-scan</td>
<td>2 (5%)</td>
<td>0</td>
</tr>
<tr>
<td>MRI</td>
<td>4 (11%)</td>
<td>11 (26%)</td>
</tr>
<tr>
<td>Angiography</td>
<td>10 (27%)</td>
<td>4 (9%)</td>
</tr>
<tr>
<td>Combinations*</td>
<td>15 (41%)</td>
<td>8 (19%)</td>
</tr>
<tr>
<td>Prior treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Embolisation</td>
<td>24 (65%)</td>
<td>1 (2%)</td>
</tr>
<tr>
<td>Re-operation</td>
<td>6 (16%)</td>
<td>7 (16%)</td>
</tr>
</tbody>
</table>

M, male; F, female. # The AMC is a referral center and patients may have had investigation in their own hospital.
*Combinations: angiography/MRI, angio/CT, angio/Doppler, MRI/Doppler or CT/MRI/angio

Table 2. The occurrence of angiogenesis, inflammation or thrombosis, and immunoexpression of tissuemarkers fibrinogen, von Willebrand factor (vWF) and CD105 in patients with high flow and with low flow malformations. Numbers between the brackets indicate percentage of patients with a positive score. NS, not significant.

<table>
<thead>
<tr>
<th></th>
<th>High flow (n=37)</th>
<th>Low flow (n=43)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angiogenesis</td>
<td>30 (81%)</td>
<td>6 (14%)</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>Inflammation</td>
<td>25 (68%)</td>
<td>15 (35%)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Thrombosis</td>
<td>12 (32%)</td>
<td>17 (40%)</td>
<td>NS</td>
</tr>
<tr>
<td>Tissuemarkers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>21 (57%)</td>
<td>32 (74%)</td>
<td>NS</td>
</tr>
<tr>
<td>vWF</td>
<td>33 (89%)</td>
<td>33 (77%)</td>
<td>NS</td>
</tr>
<tr>
<td>CD105</td>
<td>36 (97%)</td>
<td>31 (72%)</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Prior treatment strategies and microvascular growth
In 25 out of 80 patients therapeutic embolization had been performed prior to final resection of the AVM, of which 24 had high flow lesions and 1 had a low lesion (Table 3). In the embolized high flow lesions, 21 out of 24 cases (88%) showed areas of microvascular proliferative growth; in the only one low flow case in this series that was embolized, microvascular proliferation was absent.
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In the non-embolized group of A VM, microvascular growth was also observed in 27% of cases, of which 9 (69%) were high flow lesions and 6 (14%) low flow lesions. In 13 cases (6 high flow and 7 low flow) the clinical history recorded previous surgery at the same location (Table 1). In this re-operated group, 7 out of 13 (54%) showed microvascular growth compared to 29 out of 67 (43%) in A VM of patients operated for the first time.

Table 3. Occurrence microvascular angiogenesis, inflammation and thrombosis in patients treated with embolization prior to surgery (n=25) and patients who underwent no embolization (n=55). In each group the high and low flow lesions were separately categorized and scored for the histopathological parameters.

<table>
<thead>
<tr>
<th>Embolization (n=25)</th>
<th>Angiogenesis</th>
<th>Inflammation</th>
<th>Thrombosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>High flow (n=24)</td>
<td>21 (88%)</td>
<td>21 (88%)</td>
<td>8 (33%)</td>
</tr>
<tr>
<td>Low flow (n=1)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>1 (100%)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>No embolization (n=55)</th>
<th>Angiogenesis</th>
<th>Inflammation</th>
<th>Thrombosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>High flow (n=13)</td>
<td>9 (69%)</td>
<td>4 (31%) #</td>
<td>4 (31%)</td>
</tr>
<tr>
<td>Low flow (n=42)</td>
<td>6 (14%) *</td>
<td>15 (36%)</td>
<td>16 (38%)</td>
</tr>
</tbody>
</table>

*Difference in presence of angiogenesis between high flow and low flow AVM in the group of patients without prior embolization (p<0.005); # Difference in presence of inflammation between embolized and non-embolized high flow lesions (p=0.005).
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In situ inflammation and thrombosis in AVMs

Presence of inflammatory infiltrates in close proximity to the vessels of the vascular malformation was noticed in 25 out of 37 (68%) of high flow AVM and in 15 out of 43 (35%) in low flow lesions (p<0.05) (Table 2; Figure 1C). Immunophenotyping of representative cases showed that the inflammatory infiltrates were composed diffusely spread macrophages (CD68+), mast cells (tryptase+) and to a lesser extent CD3+ T-cells, and multifocal aggregates of CD79A+ B-cells (Figure 3). There was abundant expression of HLA-DR on macrophages and on a few scattered T-cells. Such inflammatory infiltrates were found in 21 out of 24 cases (88%) of the previously embolized high flow lesion. In non-embolized AVMs, inflammation occurred in 19 out of 55 (35%) cases, of which only 4 were of the high flow type.

Thrombosis was found in 12 out of 37 cases (32%) in high flow AVMs versus 17 out of 43 cases (40%) in low flow AVMs (Table 2, Figure 1D). In addition, we investigated the presence of a microvascular growth in inflamed or thrombosed lesions. There was no significant difference observed in the occurrence of inflammation or thrombosis between high flow lesion with or without angiogenesis. However, in low flow cases, inflammation occurred more frequently in lesions with (100%) than those without microvascular growth (24%) (Table 4).

Tissue markers for endothelial cell integrity and vascular damage

Extracellular immunostaining of vWF as a marker for endothelial leakage was observed in 66 out of 80 AVMs (Table 2), and was absent in all specimens of normal skin. There was no significant difference in staining between high flow and low flow lesions, but the lesions characterized by presence of microvascular growth contained more pericellular anti-vWF in both groups (Table 4; Figure 4A). There was also no
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difference in fibrinogen staining between high and low flow lesions, but the lesions without microvascular growth showed significantly more extracellular fibrinogen deposits (Table 4; Figure 4B). Endothelial expression of endoglin (CD105), interpreted as more > 5% of the vessels in the lesion being immunopositive, was observed in 84% of all cases and absent (less than 5% of vessels positive) in normal skin (n=5). Immunopositivity was significantly more frequent in high flow lesions (Table 4; Figure 4C and D). CD105 immunostaining was mainly located in the areas with proliferative angiogenesis and in the venous component of the malformation.

Table 4. Histopathologic features and immunoexpression of tissue markers (vWF, fibrinogen and CD105) in high and low flow AVM. The lesions with features of microvascular angiogenesis (+) and those without angiogenesis (-) are separately screened in each category.

<table>
<thead>
<tr>
<th></th>
<th>High flow</th>
<th>Inflammation</th>
<th>Thrombosis</th>
<th>Embolization</th>
<th>vWF</th>
<th>fibrinogen</th>
<th>CD105</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angiogenesis +</td>
<td>(n=30)</td>
<td>21 (70%)</td>
<td>10 (33%)</td>
<td>21 (70%)</td>
<td>28 (93%)</td>
<td>14 (47%)</td>
<td>29 (97%)</td>
</tr>
<tr>
<td>Angiogenesis –</td>
<td>(n=7)</td>
<td>4 (57%)</td>
<td>2 (29%)</td>
<td>3 (43%)</td>
<td>5 (71%)*</td>
<td>7 (100%)*</td>
<td>7 (100%)</td>
</tr>
<tr>
<td>Low flow</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Angiogenesis +</td>
<td>(n=6)</td>
<td>6 (100%)</td>
<td>4 (67%)</td>
<td>0/6 (0%)</td>
<td>6 (100%)</td>
<td>1 (17%)</td>
<td>6 (100%)</td>
</tr>
<tr>
<td>Angiogenesis –</td>
<td>(n=37)</td>
<td>9 (24%)*</td>
<td>13 (35%)</td>
<td>1/37 (3%)</td>
<td>27 (73%)</td>
<td>31 (84%)*</td>
<td>25 (68%)</td>
</tr>
</tbody>
</table>

* Differences in the lesional immunoexpression of extracellular vWF and fibrinogen between high flow lesions with (+) and without (-) microvascular angiogenesis (p<0.05); # Differences in presence of inflammation and fibrinogen staining between low flow AVM with (+) and those without (-) microvascular angiogenesis (p<0.05).
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Discussion

Episodes of microvascular proliferation that are associated with volume expansion of the lesion in a relatively short time span, have been observed in arteriovenous malformations (AVMs) at various topographic sites of the body.\textsuperscript{3,14,15} In this study, we noticed a significantly higher frequency of such microvascular proliferations in AVMs that were clinically classified as high flow lesions compared to AVMs with low (slow) flow characteristics, as was assessed with physical examination and supported by vascular imaging techniques in most of the patients. In the surgical resection materials of 80 patients with symptomatic AVMs, we found areas microvascular proliferation in 81\% of resections retrieved from high flow lesions versus only 14\% of the resections retrieved from low flow lesions. Histopathologically these microvascular proliferations appeared as multifocal clusters or even large sheets of immature capillary like microvessels that surrounded the large arteries and veins of the AVM. These findings suggest a clear relationship between flow forces inside the

Figure 4. A. Detail of an AVM showing a large vessel (malformed veins with thickened wall) with distinct diffuse staining with anti-vWF antibody of the vessel wall indicating endothelial leakage of vWF (asterisks). Small microvessels adjacent to the large vessel show the normal pattern of strict endothelial immunostaining with the same antibody (arrow). B. Deposits of fibrinogen in the interstitium of the AVM, stained with anti-fibrinogen antibody. C. Example of microvascular area in AVM showing endothelial immunostaining with anti-CD105 antibody of most of the microvessels; also the endothelium of a large vessel (arrow) is positive. D. Bar graphs showing the percentage of cases with positive immunostaining for vWF (A), fibrinogen (B) and CD105 (C) respectively, indicating the difference between high flow lesions (HF) and low flow lesions (LF). White area (0) is percentage without immunostaining; black area is the percentage of cases with immunostaining. There is only a significant difference with anti-CD105 antibodies between the group of HF lesions versus the group of LF lesions (p<0.05).
malformation and angiogenesis of microvessels. High flow features in these types of AVMs are caused by the presence of (usually several) fistulas between arteries and veins without interconnection of capillary beds, which leads not only to fast flow, but also to flow turbulences and altered (shear) stress in the vascular bed. In vitro studies and experimental studies have shown that flow forces in arteries and veins have an effect on vascular remodeling of pre-existing vessels, and also sprouting new vessels.\textsuperscript{4,5,16,17} In high flow AVMs, vascular remodeling appears histopathologically as tortuosity of arteries, intimal proliferations and “arterializations of veins” as has been recognized since a longtime.\textsuperscript{18} However, formation of microvascular sprouts has received much less attention. This is probably due to the fact that they occur only episodically during the long life of a malformation. Moreover, they can be recognized only histopathologically and they become manifest only in a subset of AVMs.\textsuperscript{3} Angiogenic growth factors and fluid forces cooperate in this process of vascular proliferation. VEGF, a key mediator in angiogenesis\textsuperscript{19,20} and several other angiogenic proteins have been demonstrated in other vascular diseases that are characterized by microvascular growth,\textsuperscript{21,22} so it is conceivable that they will be operative in this process of microvascular growth in AVMs. Indeed, in a previous study dealing with validation of several anti-VEGF antibodies we observed immunoexpression of VEGF in such microvascular proliferative areas in AVMs.\textsuperscript{23} The same features of an angiogenic response were also observed in some AVMs that appeared clinically as low flow lesions, albeit in much lower numbers. The extent of aberrant flow in AVMs is very variable among different lesions, and depends on the numbers and sizes of AV fistulas. It could be that the low flow lesions in which we also observed proliferating microvessels have increased flow rates, only to a limited extent, and therefore cannot be detect clinically. Still, it can be speculated that they stimulate the biomechanical pathways that evoke angiogenesis (at least on the long-term), and/or may act in combination with other external pro-angiogenic factors.\textsuperscript{24} Indeed, flow is probably not the only trigger of an angiogenic response within AVMs. Angiogenesis is an integral part of different pathological conditions like chronic inflammation, tumor growth and tissue repair, and one of the principal driving forces is hypoxia/ischemia. Therapeutic embolization, which is performed particularly in high flow AVMs creates such a state of ischemia and tissue necrosis, eventually followed by tissue repair.\textsuperscript{25} Hypoxia related proteins (HIF 1α and 2α) are upregulated in a hypoxic environment, which stimulate VEGF expression.\textsuperscript{26,27} In addition, reactive oxygen species (ROS) released in chronic inflammatory responses are also capable of activating this pathway,\textsuperscript{28} which is of importance since 50% of all lesions that we studied showed the presence of inflammatory infiltrates. A source of tissue damage and inflammation in the lesions could be therapeutic embolization that was applied in a subgroup of patients weeks prior to surgical resection.
In our study cohort, 24 patients with high flow lesions (and only one with a low flow lesion) underwent embolization, and in 88% of them indeed distinct proliferative angiogenesis was found histopathologically. Chronic inflammation was found more frequently in the embolized than in the non-embolized lesions. Endothelial endoglin (CD105) is a component of transforming growth factor beta (TGF-β) receptor complex, and thus an important player in vascular morphogenesis. Endoglin has been recognized in various pathological conditions, such as angiogenesis in tumors, wound healing and also atherosclerotic plaques, while it is hardly expressed in normal tissue. In our series, endoglin was expressed in almost all cases (97%) of high flow lesions and in 72% of low flow lesions, and not only in the areas of microvascular proliferation, but also in the larger vessels of the malformation (veins more than arteries). This suggests that also in vascular malformations these mechanisms of endothelial activation and angiogenesis are operative, even more since in control tissues of normal skin, CD105 expression is nearly absent. Extracellular diffusion of vWF as a marker of endothelial cell leakage, occurs not only in inflamed vessels, but also as a direct effect of flow related changes such as shear stress. We found this pattern of staining in a high percentage of vascular malformations, but not in normal vessels of the skin.

However, also in 9 out of 13 (69%) of the high flow AVM without previous embolization microproliferative angiogenesis was found, indicating that embolization is not the only inducer of the angiogenic response. Moreover, in the 42 low flow lesions, 36% of the lesions had an inflammatory component and 14% showed microvascular proliferations, and these lesions were never embolized because this is not considered as a therapeutic option in low flow lesions. Cytokines produced by activated (HLA-DR+) macrophages, T-cells and mast cells could be held responsible for the onset of an angiogenic response in these lesions.

The conclusion of our study is that the occurrence of microvascular proliferative response in symptomatic AVMs, which can lead to expansion of the lesion, appears to be strongly associated with clinically defined high flow characteristics of the lesions. This can be explained at least to some extent by tissue damage initiated by previous embolization and/or (related) inflammation. However, our results suggest that also the biomechanical effects of flow forces itself can play a role, since a microvascular reponse was also seen in the high flow lesions which were neither embolized, nor showed any sign of inflammation. Based on the reaction to flow characteristics and environmental influences an AVM appears to be a rather active dynamic lesion than a quiescence static lesion.
References


