Angiogenesis in congenital vascular malformations: a dynamic view on a static lesion

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Chapter 5

Proliferation and maturation of microvessels in cutaneous arteriovenous malformations – expression patterns of angiogenic and cell cycle dependent factors

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Abstract

Background: Areas of microvascular proliferation have been observed in a sub-population of symptomatic congenital vascular malformations later in life. We investigated whether this angiogenic response is followed by a stage of maturation.

Methods: Resections of vascular malformations (n=15), infantile hemangiomas (n=8) and pyogenic granulomas (n=5) were studied. Histopathologically, all lesions were screened for the presence of foci of immature and/or mature microvessels. These areas were further studied immunohistochemically for differential expression of several angiogenic factors, cell cycle dependent proteins, p53 and active caspase3. Immunostains were scored semiquantitatively.

Results: Immature microvessel areas were present in 5 vascular malformations (all of the arteriovenous type), 5 infantile hemangiomas and 5 pyogenic granulomas; these lesions also contained transitions between immature and mature microvessels. Conglomerates of mature microvessels were found in 19 cases (6 vascular malformations, 5 pyogenic granulomas and 8 infantile hemangiomas). Expression of VEGF-A, angiopoietin-1, Ki67, p16 and p21/27 ratios were overall significantly lower in mature areas than in immature areas, including those in vascular malformations. P53 and caspase3 expression was scarce in all lesions.

Conclusions: Microvascular areas in vascular malformations appear to follow the same pattern of vascular proliferation and maturation as seen in other microvascular lesions of skin and soft tissue.
Introduction

Benign cutaneous vascular lesions represent a variety of different entities with distinct clinical appearances and behavior. Infantile hemangiomas are extremely common vascular lesions. They show a typical clinical appearance of rapid postnatal growth due to lobular arranged immature microvascular masses, in which processes like vasculogenesis (at least initially) and angiogenesis appear to be involved. This proliferative stage is followed by a second stage of growth arrest, and later, partial or complete involution of the lesion. This later stages are histopathologically characterized by the presence of well-formed, often dilated microvessels lined with flat endothelium, interpreted as vascular maturation. Similar patterns of microvascular proliferation followed by maturation can be observed in organizing thrombus and in pyogenic granulomas, which are often complicated by ulceration in the proliferative phase. These are both considered as reactive phenomena. Vascular malformations on the other hand are congenital vascular anomalies composed of mature blood and/or lymphatic vessels lined with quiescent flat endothelium. They have a typical growth pattern of a slowly progressive enlargement of the lesion over many years. Despite this indolent type of behavior, solid sheets of immature but benign microvessels with proliferative characteristics have also been observed in such congenital malformations, especially in the arteriovenous forms (AVM). The nature of such proliferative masses in AVMs is at present unclear, and specifically it is not known whether they occur as an integral part of the malformation itself, or alternatively they could represent reactive vasoproliferations due to stimuli such as hypoxia or trauma. However, hypothetically the vasoproliferative foci in AVMs may undergo a similar pattern of maturation as seen in other benign vascular lesions, and hence contribute to the hamartomatous mass of vessels and associated matrix tissue that is characteristic for AVMs. Such a feature could explain the episodes of disproportionate growth that have been described in AVMs. We investigated 15 vascular malformations in comparison to 8 infantile hemangiomas and 5 pyogenic granulomas which were histopathologically screened for the presence of vasoproliferative foci as well as conglomerates of maturated microvessels, using the same histomorphological criteria in all lesions. To complete the study we compared in these distinct microvascular areas the in situ expression and immunolocalization of several angiogenic and cell cycle dependent proteins.
Microvascular proliferation and maturation in arteriovenous malformations

Materials and Methods

Specimen selection and histopathology
Surgical specimens of clinically and histopathologically well documented vascular lesions of skin and/or subcutis were retrieved from the archives of the Pathology department of the Academic Medical Center in Amsterdam. The materials had been fixed in buffered formalin, routinely processed and embedded in paraffin. Hematoxylin and Eosin (H&E) and Elastic van Gieson (EvG) stained tissue sections were reviewed to classify the cases following the recommendations of the International Society for the Study of Vascular Anomalies (ISSVA). Accordingly, a total of 28 specimens were selected of which appropriate clinical data was available for final diagnosis: 15 cases of vascular malformations (10 of the arteriovenous type; AVM and 5 of the venous type; VM), 8 cases of infantile hemangioma (IH) and 5 cases of pyogenic granuloma (PG). Infantile hemangiomas and pyogenic granulomas were located in the skin only, but several vascular malformations also extended into the deeper soft tissues. Anti-glucose transporter type 1 (GLUT1 antibody, Dako) immunohistochemistry was used to confirm the diagnosis of infantile hemangioma. To differentiate between lymphatic and blood vessel endothelium, all sections containing microvascular areas, were immunostained with anti-D2-40 (Immunologic) which stains specifically lymphatic endothelium. The Wilms tumor 1 (WT1) protein has recently reported to be expressed in vascular neoplasms, but not in vascular malformations. Therefore we also applied anti-WT1 (Thermo RB-9267) immunostaining in order to study the expression of WT1 protein in the microvascular areas of vascular malformations, infantile hemangiomas and pyogenic granulomas.

Definition of immature and mature microvascular areas
H&E sections were screened in combination with anti-CD31 (endothelial cells) and anti-SMA-1 (pericytes/smooth muscle cells) immunostained sections for determination of foci of immature and/or mature microvessels within the lesions. Immature microvessels were defined on H&E sections as solid appearing areas of closely packed capillary vessels with inconspicuous lumina and large swollen endothelial cells, and which contained in the adjacent immunostained sections an interrupted or continuous layer of SMA-1 positive cells (pericytes) and CD31 positivity of the swollen endothelial cells. Mature microvessels were defined on H&E sections as structures with dilated lumina, and which contained in the adjacent immunostained sections 1-2 layers of SMA-1 positive cells (pericytes/smooth muscle cells) and CD31 positivity of the flat endothelium.
**Immunohistochemistry and monoclonal antibodies**

Adjacent serially 4mm sections of all lesions were immunostained with antibodies reactive with:

1. Major angiogenic proteins; Vascular Endothelial Growth Factor-A (VEGF-A, rabbit polyclonal RB-9031, ThermoFisher Scientific, Fremont, CA, USA),16 Angiopoietin-1 (Ang-1) (rabbit polyclonal, Chemicon/Millipore, Temecula, CA, USA), Angiopoietin-2 (Ang-2) (goat polyclonal, Chemicon/Millipore) and Tie2 tyrosine kinase receptor (Tie2) (rabbit polyclonal, Chemicon/Millipore).

2. Major cell cycle dependent proteins; Ki67 (rabbit SP6, ThermoFisher), p16 (p16 INK4a, mouse E6H4, Dako, Glostrup, Denmark), p21 and p27 (p21 WAF1-CIP1, mouse SXII8 and p27 Kip1, mouse SX53G8, Dako), which have previously described to be differentially expressed in endothelial cell turnover.17,18

3. Marker for apoptosis; active caspase-3 (rabbit polyclonal, Cell Signaling, Danvers, MA, USA)

4. Tumor suppressor gene product; p53 (rabbit SP5, ThermoFisher)

Immunoreactivity of GLUT1, D2-40, WT1, CD31, VEGF-A, Ang-1, -2, Tie2 and p53 antibodies was visualized in immunosingle stains. Briefly, the sections were dewaxed in xylene, rehydrated and endogenous peroxidase activity was blocked in 0.3% H₂O₂ in methanol for 20 min. Tissue pre-treatment was performed with heat-induced epitope retrieval (HIER) using Tris-EDTA pH 9.0 or citrate 6.0 (in case of GLUT1, VEGF-A). Immunoreactivity was visualized by diaminobenzidine tetrachloride (DAB), (GLUT1, D2-40, WT1, VEGF-A) or Vector Red (Vector laboratories, Burlingame, CA, USA) (CD31, Ang-1, Ang-2, Tie2).

Double staining methods were applied to assess cell specific immunolocalization of Ki67, p16 and caspase3 antibodies on endothelial cells and/or pericytes. A Ki67/SMA immunodouble staining was performed as previously described with Ki67 antibody visualised by DAB in brown, followed by application of anti vascular smooth muscle antigen (anti-SMA-1) visualised by Vector Red.8 P16/SMA, caspase3/SMA, p21/p27 immunodouble stains were performed with the sequential alkaline phosphatase double staining method using Vector Red and Vector Blue as chromogens.19 The p27/p21 ratio of immunopositivity in all lesions was analysed with the use of Nuance 3.0 Spectral Imaging (Caliper Life Sciences, Hopkinton, MA, USA), a computer assisted optical technique which unmixes colors in the section based on their spectral characteristics, and allows a detailed analysis of double stained cells.20,21

For GLUT1 and WT1 the immunoreactivity on infantile hemangioma was taken as a positive reference, according to the literature.12,15 Normal placental tissue and palatinal tonsil tissue, treated according the same protocol, were used as positive control for all other antibodies.
**Semiquantitative evaluation of immunostains**

Immunostains were semiquantitatively scored in order to compare patterns of immunoreactivity between areas of immature and mature microvessels in all types of lesions.

GLUT1, WT1 and D2-40 were screened as either positive or negative on the endothelium of vessels.

Ang-1, Ang-2, VEGF-A and Tie2 receptor antibodies showed distinct differences in the intensity of immunostaining among lesions, and were scored as follows: 0: absent; 1: positive staining of (almost all) vessels with low color intensity; 2: positive staining with high color intensity.

P16, Ki67, p53 and caspase3 antibody reactivity was scored on the percentage of vessels containing immunopositive (+ve) cells: 0: 0-5% +ve, 1: 5-10% +ve, 2: 10-50% +ve, 3: >50% +ve.

Reactivity of anti-p27 and p21 antibodies was scored as p21/p27 ratio of immunostaining in each section with the use of spectral image analysis. Since p27 staining exceeded by far p21 staining in nearly all cases, we applied the following score: 1: p27 >>> p21 (p21 staining scarce to absent of <1%), 2: p27 >> p21 (p21 staining 1-10%), 3: p27 > p21 (p21 staining 10-50%), 4: p21 staining > 50%.

**Reverse Transcription-Polymerase Chain Reaction, RT-PCR.**

Frozen tissue biopsies of 21 vascular malformations were used for RT-PCR. Of each biopsy 2 sections were cut and stained with H&E and EvG for morphological assessment. RNA was extracted from the material using TRIZOL (Invitrogen, Breda, the Netherlands) according to the instructions by the manufacturer. cDNA was synthesized from 5µg total RNA using oligo dT as primer and M-MLV reverse transcriptase (Invitrogen). cDNA was amplified by PCR in a reaction mixture (25µl) containing Taq-buffer (Invitrogen), 1.5mM MgCl\textsubscript{2}, 0.2mM dNTPs, 5pmol of each 5’ and 3’ primer and 0.25U Taq DNA polymerase (Invitrogen). The following primers were used: Ang-1 forward: 5’-TGCAGAGAGATGCTCCACAC 3’ and reverse: 5’-AGCTTTCTGGTGCTCTGC-3’; Ang-2 forward: 5’-GGGAAGGGAATGAGGCTTAC-3’ and reverse: 5’-AAGTTGGAAGGACCACATGC-3’; VEGF-A forward 5’-GGGCAGAATCATCAGAAGT-3’ and reverse: 5’-ATCTGCATGGTGATGTTGGA-3’; Beta-actin: forward: 5’-CCTTCCTGGGCCATGGAGT-3’ and reverse: 5’-GCTCAGGAGGAGCAATGATCT-3’.

After 35 cycles on a Dyad thermocycler (Bio-Rad, Hercules, CA, USA) aliquots of PCR products were electrophoresed on a 1.0% agarose gel and visualized by
ethidium bromide staining. A mock PCR (water instead of cDNA) was included to exclude contamination in all experiments.

Statistical analysis
Semiquantitative scores of immunohistochemical expression of all antibodies were subject to statistical analysis using PASW statistics. The results of immunostaining were expressed as mean ± standard deviation (SD). For comparison of immunoreactivity in the immature and the mature microvascular areas of all lesions we used the non-parametric Mann-Whitney U test. P-values <0.05 were considered statistically significant.

Results
Of the 15 vascular malformations that were included in this study, 5 cases showed multifocally distinct areas immature capillary vessels (solid growth, inconspicuous lumina, swollen endothelium), which were all of the arteriovenous type of malformation (AVM) (Figure 1A and B). All these AVMs also had conglomerates of mature microvessels with well outlined vascular lumina lined with flat endothelium (Figure 1C). Immunohistochemically, both these immature and mature types of microvessels had a luminal endothelial component and a clear pericyte/smooth muscle cell lining, visualized with anti-CD31 and anti-SMA-1 antibodies (Figure 1D and E). Only one vascular malformation, classified as a venous malformation (VM), showed presence of areas composed of only mature capillaries in close connection to the cavernous veins; two other VMs were complicated by containing old thrombus masses with growth of fibrovascular sprouts. Five out of 8 infantile hemangiomas and all pyogenic granulomas showed a same morphologic pattern of both areas with immature and mature capillary microvessels, albeit arranged in more lobular patterns, whereas 3 infantile hemangiomas were only composed of mature microvessels. Endothelial cell immunostaining of GLUT1 was positive in the eight infantile hemangiomas only. Adjacent anti-D2-40 immunostained sections showed relatively sparse numbers of lymphatic vessels in all cases of vascular malformations, and absence of lymphatic vessels in the conglomerates of both immature and mature microvessels mentioned above. Anti-WT1 uniformly stained the microvascular areas of all lesions, including those in vascular malformations. Notably, there were also no differences in WT1 immunostaining between the immature and mature microvesssels of the lesions (Figure 1F and G).
Microvascular proliferation and maturation in arteriovenous malformations

Figure 1. A. Overview of an arteriovenous malformation, in which the pre-existing vessels of the malformations (arrows) are completely surrounded by solid sheets of immature microvessels (asterisks). Also some pre-existing adipocytes are present (for example upper right) (H&E stain). B. Detail of a proliferative area showing small vessels with inconspicuous lumina lined with endothelium (H&E stain). C. Detail of a mature microvessel area (taken from the same AVM, but not visible in Figure A) (H&E stain). D and E. Anti-SMA-1 immunostains of a proliferative (D) and mature (E) microvascular area showing a pericyte lining in both instances, but which is clearly outlined better in the mature microvessels (anti-SMA-1 immunostain, visualized with Vector red). F and G. Anti-WT1 immunostains of a proliferative (F) and mature (G) microvascular area showing distinct immunostaining of endothelial cells in both types of vessels (anti-WT-1 immunostain, visualized in brown with DAB).
Microvascular proliferation and maturation in arteriovenous malformations

Figure 2. RT-PCR analysis showing the expression of 3 major angiogenic proteins in frozen tissue biopsies of AVMs with either absence (left) or presence (right) of a microvascular proliferative intrallesional component.

Table 1. Results of immunohistochemical staining of angiogenic growth factors and cell cycle dependent proteins, caspase3 and p53. Panel A. Solid sheaths of immature microvessels; Panel B. Mature microvessels.

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<th>Ang2</th>
<th>Tie2</th>
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IH, infantile hemangioma; PG, pyogenic granuloma.

Counts in each group represent the sum of the semiquantitative scores of all individual cases (see methods section) divided by the total number of cases.

¹ Mainly stromal cell surrounding capillaries are positive; ² stromal cells are negative; *p<0.005. Immature (A) vs mature (B) microvessels (Mann Whitney U test).
**mRNA expression of angiogenic proteins**

Tissue for PCR was sampled from 21 vascular malformations from which frozen material was available. Of these, 10 contained proliferative areas and 11 were devoid of such proliferative features at histology. Interestingly Ang-1, Ang-2 and VEGF-A mRNA could be detected in both proliferative areas as well in malformations without microproliferative angiogenesis. As shown in Figure 2, which shows all individual data, there were no clear differences in presence of angiogenic proteins between both groups, since most lesions did express mRNA for all three angiogenic factors.

**In situ protein expression**

The immunolocalization of proteins that could relate to a process of vascular proliferation and maturation were studied with the use of immunosingle and -double stains in histopathologically defined immature and mature areas of all lesions. For each stain a semiquantitative grading of the staining results was applied, which is summarized in Table 1. Overall, immunoreactivity appeared significantly higher in immature than in mature areas for all proteins, except for Ang-2. However, some differences in immunostaining patterns could be observed among the individual groups of vascular lesions.

Angiogenic proteins (Figure 3): VEGF-A immunostaining was seen in all the investigated cases, but with a clearly higher staining intensity in proliferative immature areas than in mature microvascular areas of all lesions. Anti-Ang-1 immunopositivity was found in proliferative areas of all lesions, except for PG, and to a much lower extent in the mature microvessels of the same lesions. Also the immature microvessels in two cases of VMs with organizing thrombus appeared clearly anti-Ang-1 positive. Conversely, anti-Ang-2 staining was found positive in mature microvessels in infantile hemangiomas, whereas all others were completely negative. Tie2 staining was seen in both the immature and in the mature areas of nearly all lesions.

Cell cycle dependent proteins (Figure 4A-D): Areas of immature proliferative angiogenesis showed distinctly more p16 immunopositivity. AVMs showed slight to diffuse (only one case) positive expression of p16 in endothelial cells, whereas the expression in the newly formed capillaries in the organized thrombus material, infantile hemangiomas and pyogenic granulomas were moderate to diffuse positive in endothelial cells. In addition, there was a prominent immunostaining of stromal cells surrounding the immature capillaries in AVMs, thrombus and pyogenic granulomas, but not in infantile hemangiomas (Figure 4B). Ki67 immunostaining showed an increase in proliferative activity, with highest expression in PG lesions. Only slight expression was seen in mature capillaries in 2 out of 6 vascular malformations and 5 out of 8 infantile hemangiomas. All pyogenic granulomas showed slight to moderate expression. In the evaluation of the ratio p21/p27 in all lesions there was more p21 expressed in the immature capillaries compared to the mature capillaries (Figure 5A-C).
Figure 3. Representative examples of immunostaining patterns of anti-angiogenic growth factor antibodies in immature (left panel) and mature (right panel) microvessels (mv), illustrating the semiquantitative scores that were used in this study. A. VEGF-A immunostain of immature mv area in an infantile hemangioma, score 2 (high intensity of all vessels). B. VEGF-A immunostain of mature mv area in an infantile hemangioma, score 1 (low intensity of all vessels). C. Ang-1 immunostain immature mv area in an AVM, score 1 (low intensity of all vessels). D. Ang-1 immunostain of mature mv area in an infantile hemangioma, score 1 (low intensity of all vessels). E. Ang-1 immunostain of immature microvessels in thrombus, score 1 (low intensity of all vessels). Yellow stained material represents hemosiderin pigment. F. Ang-2 immunostain of immature mv in thrombus material, score 0 (negative). G. Tie2 immunostain of immature mv area in an AVM, score 1 (low intensity of all vessels). H. Tie2 immunostain of mature mv area in an AVM, score 2 (high intensity of all vessels).
Figure 4. Representative examples of immunostaining patterns of antibodies reactive with cell cycle dependent proteins, caspase3 and p53 in immature (left panel) and mature (right panel) microvessels (mv), illustrating the semiquantitative scores used in this study. A. P16 immunostain of immature mv area in an infantile hemangioma, score 3 (>50% of vessels positive). B. P16 immunostain of mature mv area in an AVM, score 0 (nearly all endothelial cells are negative, only perivascular cells stain positive). C. Ki67 immunostain of an AVM (immature microvessels), score 2 (10-50% of vessels positive). D. Ki67 immunostain of an AVM (mature microvessels), score 0 (<5% of vessels positive). E. Caspase3 immunostain of an AVM (immature microvessels), score 0 (<5% of vessels positive). F. Caspase3 immunostain of an infantile hemangioma (mature microvessels), score 0 (<5% of vessels positive). G. P53 immunostain of an AVM (immature microvessels), showing 1 positive nucleus (score 0; <5% of vessels positive). Note that only strongly positive nuclei are considered positive. H. P53 immunostain of an AVM (mature microvessels), score 0 (<5% of vessels positive). Positive nuclei were never observed in mature microvessels in this study.
There was more expression of p21 in AVMs, organized thrombus material and pyogenic granuloma compared to infantile hemangioma. The areas of proliferative angiogenesis in infantile hemangioma showed much less expression of p21 (score 1). All types of mature vessels (arteries, veins or capillaries) show almost exclusively expression of p27 and hardly any expression of p21 (Figure 5D-F).

P53 and active caspase3 (Figure 4E-H): Anti-p53 and -caspase3 immunopositivity appeared as a nuclear immunostaining pattern. P53 and caspase3 expressions were low (<5% of cells) to absent in the immature and mature areas of all types of lesions.
Microvascular proliferation and maturation in arteriovenous malformations

Discussion

The most frequently observed benign vascular lesions during infancy and adolescence, infantile hemangiomas and pyogenic granulomas, show a biphasic growth pattern of proliferative growth of microvessels followed by maturation or even regression of vessels.22 In congenital vascular malformations, which are by definition composed of malformed but mature vessels and show a slowly progressive growth pattern during lifetime,6 such episodes of microvascular angiogenesis have been described only recently and in particular in the arteriovenous subtype of vascular malformations.8,23 In this study, we demonstrated that this pattern of microvascular proliferation occurred in a subgroup of vascular malformations, which were all of the arteriovenous type, and follows the same pattern of microvascular maturation as is seen in infantile hemangiomas and pyogenic granulomas. The lesions encountered in this study, which contained histopathologically defined vasoproliferative areas (5 malformations of the arteriovenous type, 5 infantile hemangioma and 5 pyogenic granuloma), also contained the same transitional patterns between immature and mature vessels. Moreover, similar areas with conglomerates composed of only mature microvessels were present in 5 pyogenic granulomas and 8 infantile hemangiomas and also in 6 of the 15 vascular malformations (specifically, all 5 arteriovenous malformations that also had immature vessel areas, and in addition one venous malformation). With the use of WT1 immunostaining, recommended to distinguish between angioma (WT1 positive endothelial cells) and vascular malformations (WT1 negative endothelial cells, except for arteriovenous malformations),24 we found no difference in staining pattern between either the proliferative vascular areas or the mature vascular areas of all types of lesions (all showing uniform endothelial cell positivity). In line with these histopathological observations, we found that the expression of the proliferation marker Ki67 was overall, but also among all individual groups, significantly higher in the areas with solid sheets of immature capillary vessels than in those with well outlined microvessels. This supports the notion of microvascular maturation and stabilization. Remarkably, in all cases which had a component of high proliferative activity as indicated by Ki67 staining, there was also pronounced immunostaining of p16 in the same tissue areas. Absence of Ki67 staining in the walls of mature microvessels corresponded with scarce or absent p16 immunostaining in the same vessels. P16 is a tumor suppressor protein which has, aside from controlling the cell cycle in many other tissues, also anti-migratory and anti-angiogenic effects associated with vessel formation. It acts in a VEGF dependent fashion, and is localized in the lamellipodia of endothelial cells, structures that are required for spreading and migration of the cells.25,26 P16 is not expressed in normal arteries, but transient expression has been observed in experimental models of vascular injury followed by regeneration of the injured ves-
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sels, which fits with our observations of p16 positivity in proliferative areas and absent staining in areas with low Ki67 expression on microvessels. Similar to p16 protein, cyclin-dependent kinase inhibitors p21 and p27 also control the order of the cell cycle which is required for homeostasis between growth and cell death. P27 has a major function in stopping or slowing down the cell division cycle and is highly expressed in non-proliferating, quiescent cells, including normal arteries. Shortly after arterial injury p27 appears to be downregulated, with subsequent recovery of expression in the later phases of arterial repair. Conversely, the same studies have shown that p21 is hardly expressed in the quiescent cells of normal arteries. In our materials, p27 showed by far the most dominant expression pattern over p21, which was confirmed by spectral analysis of p21/p27 immunostaining of all lesions. However, still significantly higher ratios of p21/p27 were observed in the immature proliferative areas than in mature areas of all types of lesions that we investigated, including microvascular masses in vascular malformations. Overall, our results suggest a trend towards vascular stabilization (indicated by high or almost exclusive p27 expression) in all these lesions. P53 is well known as a tumor suppressor gene, but is also reported to be active in the control of angiogenic responses. P53 expression has been described in pyogenic granulomas and particularly high rates of expression have been noted in vascular malignancies, i.e. angiosarcomas. In our series p53 positivity was seen only in a few cells (<5%), and only in areas with immature vessels, whereas mature vessels were totally negative in p53. Similarly, apoptotic rates as shown by anti-active caspase3 reactivity were very low to absent in the areas with mature microvessels.

Since angiogenesis is characterized by the release of several angiogenic growth factors, one could speculate that distinct patterns of morphology and clinical behavior of vascular skin lesions relate to distinct patterns of expression of such angiogenic proteins. Many factors modulate microvascular growth, but the VEGF family members and angiopoietins are believed to act almost exclusively on endothelium. Ang-1 has pro-angiogenic effects and promotes the integrity of the endothelial lining of vessels in synergy with VEGF-A, whereas Ang-2 destabilizes endothelial cells (ECs) and promotes regression of vessels, especially when VEGF levels are low. The only lesion that showed a distinct pattern in expression of angiopoietins and Tie2 receptor in our series was infantile hemangioma. Most studies on angiogenic growth factors in the skin have been carried out on infantile hemangioma, the most prevalent vascular tumor in children, and which invariably also showed up-regulation of VEGF in proliferative phase but not in the involuting stage of the lesion. Yu et al and Calicchio et al found an increase in mRNA expression of Tie2 in endothelial cell cultures derived from infantile hemangioma corresponding to an enhanced cellular response to Ang-1 and down-regulation of Ang-2, which is largely in line
with our findings on the immunostaining pattern of infantile hemangioma. Also in pyogenic granulomas, which are considered to arise due to stimuli such as trauma, the expression of VEGF and angiopoietins, especially in areas of proliferating endothelial cells has been reported earlier.\textsuperscript{40-42} And, in agreement to what we found, immunostaining for VEGF was less marked in more mature vessels. Immunoreactivity of these angiogenic proteins in microvessels in vascular malformations have not been described earlier in the literature. However, as we have shown in this study, they display comparable expression patterns as are found in other vascular lesions with microvascular areas.

In conclusion, the microvascular proliferative responses that may complicate the natural course of a subgroup of arteriovenous malformations appear to follow the same pattern of vascular maturation as seen in other microvascular lesions of skin and soft tissue. The trigger of this angiogenic response has to be explored in further studies, but hypoxia and/or inflammatory cytokines such as TNF and IL-1β, which are also be involved in the pathogenesis of infantile hemangiomas\textsuperscript{43} and pyogenic granulomas, will be likely candidates. It is certainly true that angiogenesis cannot explain all the disproportionate growth of symptomatic arteriovenous malformations; in fact, several other factors are known to have an effect on the growth of lesions, which are the formation of collaterals, dilatation and thickening of veins, or inflammation and ulceration. It is also not known why microvascular proliferative responses occur almost solely in arteriovenous malformations and not in other types of malformations, such as the venous types, which are more often complicated by thrombosis and inflammation.\textsuperscript{44} Nevertheless in addition to the complications mentioned above, a microvascular proliferative response has been associated also with the onset of symptoms in patients.

Nowadays many efforts are put now into inhibition of angioproliferative responses in hemangiomas, especially in those who are symptomatic due to ulceration or compression of adjacent structures.\textsuperscript{45} A similar approach could be followed in the treatment of vascular malformations, specifically the arteriovenous types, that are complicated by (episodic) microvascular proliferations.
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