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

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Chapter 13

Summary and discussion

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This study was initiated by a case of a 40-year old male, who noticed sudden expansive growth in the malformation of which he was born with. Histopathology revealed an unexpected extensive microvascular proliferation adjacent to the large malformed vessels of the arteriovenous malformation (AVM). This remarkable finding of vasoproliferative growth is something we knew from infantile hemangiomas or reactive vascular lesions, like pyogenic granulomas, but not of an arteriovenous malformation. Further cases of AVMs with similar microvascular proliferative responses prompted us to investigate the nature of this process. Such insights can provide information on how these lesions should fit in the current classification of vascular lesions and how they should be treated properly.

Part I. Vascular lesions and angiogenesis – an overview

Chapter 2. Benign vascular lesions of skin and soft tissue - classification.
Vascular malformations are considered as congenital anomalies that result from local errors in angiogenic development during embryogenesis.[1] Vascular malformations should be clearly distinguished from other benign vascular lesions such as the widely spread infantile hemangiomas (IHs), which are the most common vascular tumors of children. This is because malformations behave different from vascular tumors in terms of their growth characteristics, and therefore require other treatment strategies. However, in the clinical arena they are also the lesions that lead to confusion when it comes to a proper nomenclature and classification.[2,3] Often, the suffix '-oma' is given to vascular lesions that in fact should be classified as vascular malformations. For the reasons set above, we performed a review of historical and current insights in the classification of vascular anomalies of skin and soft tissue. The early biological classification of Mulliken and Glowacki[4] is mentioned, because this classification was an important step towards a dichotomy between vascular tumors and vascular malformations and formed the basis for a classification that is currently recommended by the International Society for the Study of Vascular Anomalies (ISSVA).[5] Also other classifications, particularly those who are forwarded in the major textbooks of soft tissue tumors as they are used in daily practice by surgical pathologists are reviewed in the light of the above. In the second part of this chapter an overview is given of the different types of vascular malformations and hemangiomas or related entities as far as they are included in the ISSVA classification. In this review we recognized the importance of a correct classification and differential diagnosis that must be based on a combination of proper histopathological diagnosis with helpful tools, like GLUT1 immunohistochemistry[6], in combination with appropriate clinical and vascular imaging data.

For the purpose of this thesis, it was of pivotal importance that we handled strict diagnostic criteria for the cases that we incorporated in the study, particularly the vascular malformations. Therefore, we used the ISSVA classification as the basis for inclusion of patients, which has resulted in one of the largest series of clinically *and* histopathologically well documented congenital vascular malformations worldwide. Although the ISSVA classification was mainly designed by the Working Group to classify vascular anomalies of children and adolescents, it is in our experience also useful to denominate vascular malformations in adults. It is certainly true that not all vascular lesions, especially those in adults, fit properly in the classification. For example, recently recognized new lesions, like cutaneous epitheloid angiomatous nodule, acquired elastotic hemangioma and some others are not easy to classify as either a tumor or malformation with the current knowledge (and uncertainties) on their etiology and biological behavior.[7]

Chapter 3. Angiogenesis in vascular lesions – basic aspects.

The onset of microvascular growth in AVMs, to such an extent that it can lead to symptomatic lesions at least in a subpopulation of the patients, is the main subject of this thesis. In this chapter we reviewed the current insights in the involvement of vasculogenesis, angiogenesis and/or arteriogenesis in onset and growth of benign vascular lesions of skin and soft tissue. It turns out that the best studied lesions, also in terms of pathophysiology, are infantile hemangiomas which are the most common vascular lesions in young children. Infantile hemangiomas have an intriguing growth pattern and also unique distinguishing features, such as the still somewhat mysterious GLUT1 immunostaining in this type of angioma and the presence of large numbers of mast cells.[6,8] Several studies pointed out that processes of vasculogenesis, at least initially, and also angiogenesis are involved[9], in which many angiogenic growth factors and growth inhibitors are involved. Still it remains uncertain why exactly the lesions regress by the time and what specifically are the driving forces.

The focus of our study, congenital vascular malformations, appears to be the least studied vascular anomalies, and especially those that occur extracranially. It could be that the interest for angiogenic processes in these lesions lags behind, because they have been considered since a long time as rather static, very slowly growing lesions. Most of the recent insights on the vasculogenic and/or angiogenic aspects of malformations are investigated in cerebral lesions. There is growing evidence suggesting that in cerebral AVMs processes of active angiogenesis and vascular remodeling interact, leading to a dynamic process of episodic growth and regression, also in adult life.[10] The results of our study (see later) support a concept that the same applies for extracranial AVMs.

Part II Angiogenesis in vascular malformations

Chapter 4. Microvascular proliferation in congenital vascular malformations of skin and soft tissue.

It is known that episodic volume expansion in arteriovenous malformations can occur during puberty or pregnancy or after trauma, probably due to dilatation, collateralization and/or thickening of vessels. However, the extensive microvascular proliferation in a vascular malformation that we found in the index case of our studies (Chapter 1) could also be an explanation for the expansive growth. In this study the features of microvascular proliferation were systematically investigated in 107 resection specimens of clinically and histopathologically well-documented vascular malformations. They 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). We observed in 30% of all vascular malformations the occurrence of microvascular proliferation amidst the vessels of the malformation, of which 94% appeared to be of the arteriovenous type of malformation. In a subset of these, the vasoproliferative growth extended into pre-existing surrounding adipose tissue or skeletal muscle. MVD and MCD were significantly higher in areas of microvascular proliferation, with high Ki67 labeling indexes in both ECs and VSMCs, compared to areas with mature vessels. Age, sex and location of vascular malformation had no predictive value for the occurrence of microvascular proliferation. Another very interesting finding was that such vasoproliferative phenomena (in terms of area involvement as well as Ki67 labeling indexes of ECs and VSMCs) were far more extensive in men than in women, although the underlying mechanisms are difficult to explain at present.

Chapter 5. Proliferation and maturation of microvessels in cutaneous arteriovenous malformations – expression patterns of angiogenic and cell cycle independent factors.

The vasoproliferative growth, as described in Chapter 4 was histomorphologically indiscernible from that of infantile hemangiomas and pyogenic granulomas. These benign vascular lesions are different entities with their own typical clinical appearance, but in their behavior and histopathologically they show the same biphasic course of proliferation followed by maturation of microvessels. To further analyze the nature of the microvascular proliferative response in vascular malformations we investigated whether it is also followed by maturation. Histopathologically, resections of 15 vascular malformations (10 arteriovenous, 5 venous), 8 infantile hemangiomas and 5 pyogenic granulomas were screened for presence of foci of immature and/or mature

microvessels. Anti-GLUT1 and anti-WT1 immunohistochemistry were used in the differential diagnosis. Immunohistochemical analysis of both the immature and mature areas was performed, using several angiogenic factors, cell cycle dependent proteins, p53 and active caspase3. In 5 arteriovenous malformations, 5 infantile hemangiomas and 5 pyogenic granulomas we observed immature microvascular areas, also with a transitional pattern of maturation. Conglomerates composed of mature microvessels were found in 19 cases (6 vascular malformations, 5 pyogenic granulomas and 8 infantile hemangiomas). VEGF-A, Angiopoietin-1, Ki67, p16 and p21/27 ratio were overall significantly higher in the areas composed of immature microvessels versus the mature areas. There were no clear differences among the individual groups, including arteriovenous malformations, indicating that arteriovenous malformations follow the same pattern of proliferation and maturation as seen in infantile hemangiomas and reactive pyogenic granulomas. Also with the use of WT1 immunostaining, which is additionally used to distinguish between angioma (WT1 positive endothelial cells) and vascular malformations (WT1 negative endothelial cells, except for arteriovenous malformations)[11], we found no difference in staining pattern between either the immature vascular areas or the mature vascular areas of all types of lesions, since all showed an uniform endothelial cell positivity. The notion of microvascular maturation and stabilization was strengthened by expression of other cell cycle dependent proteins, like p16 and p21/p27 ratio in areas with microvascular proliferative growth.

Chapter 6. Anti-human Vascular Endothelial Growth Factor (VEGF) antibody selection for immunohistochemical staining of proliferating blood vessels.

The reliability of commercially available antibodies in terms of specificity and applicability for immunohistochemical purposes in diagnostic pathology or in biomedical research is of great importance, because it is obviously clear that conclusions are (partly) based on the outcome of immunoexpression patterns. When the presence and/or localization for a specific epitope needs to be identified in a tissue, there is usually a choice of several different antibodies from different suppliers available. It is often difficult to distract from research papers how exactly one of the antibodies was selected as the antibody of choice. Proper immunostaining of vascular endothelial growth factor antibody (VEGF-A), a key regulator in angiogenesis[12,13] was of pivotal importance for our studies, but we had to make a choice out of nine commercially available antibodies, which seemed all useful at first instance.

First, all antibodies were optimized for their performance in immunohistochemistry, with placenta and colon adenocarcinoma as positive control tissues. Five antibodies were regarded as unfit for VEGF immunostaining based on poor immunostaining results when they were applied on positive control tissues. Subsequently, Western

blot analysis using VEGF rabbit polyclonal antibody (Thermo RB-9031) revealed a clear 45-kDa band in tissue extracts from placenta and immature vessels of vascular malformations and, in combination with excellent immunostaining of positive controls, Thermo RB-9031 was therefore considered as reliable. This study highlights the importance of testing more than one antibody before starting immunohistochemical analysis in a large study design.

Chapter 7. Microvascular proliferations in arteriovenous malformations relate to high flow characteristics, inflammation and previous therapeutic embolization of the lesion.

Vascular malformations are clinically categorized according to their hemodynamic features as low (slow) or high (fast) flow lesions. High flow lesions are always of the arteriovenous type of malformations. We investigated whether angiogenic responses in AVM relate to these different flow characteristics. Resection specimens of 80 histopathologically proven AVMs were clinically categorized as either high or low flow lesions, and histopathologically screened for the presence of microvessels, inflammation and/or thrombosis. Of all lesions, 37 AVMs were clinically classified as high flow lesions and 43 as low flow. In 81% of the high flow lesions microvascular proliferations were seen versus 14 % of the low flow lesions. In high flow lesions, which were embolized prior to surgery (30% of all AVM cases), 88% showed microvascular proliferation, 88% inflammation and 33% thrombosis. Embolization and (related) inflammation causes a hypoxic environment which can be a trigger for angiogenesis. However, similar vasoproliferative responses were also observed in AVM without prior embolization (69% high flow and 14% low flow lesions). Immunohistochemistry was performed with anti-endoglin antibody (anti-CD105), because endoglin expression is up-regulated in various pathologic conditions, such as angiogenesis in tumors, wound healing and also atherosclerotic plaques, and there for considered as a marker for endothelial activation[14-16] In our series, endoglin was expressed in almost all cases (97%) of high flow lesions and in 72% of low flow lesions. Its expression was found not only in the areas of microvascular proliferations, but also in the larger vessels of the malformation. This suggests that also in vascular malformations these mechanisms of endothelial activation and angiogenesis are operative. Immunostained extra-endothelial depositions of von Willebrand factor (as a marker of vascular leakage[17]) were also found in most AVMs, although not in all the vessels of the malformation. These findings indicate that microvascular proliferative masses in AVMs are positively associated with high flow characteristics. This could be explained by tissue damaging effects of previous embolization and/or (related) inflammation only in a subset of the lesions, leaving a role for the biomechanical effects of high flow in this process.

Traditionally vascular malformations are slowly progressive lesions that grow proportionally with the life of the child, and they are composed of mature large vessels lined with quiescent endothelial cells. Our finding of an angiogenic response in 30% of all vascular malformations contrasts markedly with the original view of considering vascular malformations as static lesions. All the patients who were included in our study had serious clinical symptoms, since the angiogenic response that we found in the tissue appeared to be so extensively that it could relate to a mass forming effect, we suppose that it could also relate to clinical symptoms.

Nearly all of them were AVMs, of which is known that they have aberrant flow characteristics due to presence of AV-fistulas. Hemodynamic forces can initiate or aggravate an angiogenic response, at least in experimental studies.[17,18] And indeed, histopathological features of microvascular growth were found in more than 80% of lesions that were clinical characterized as high flow lesions, which suggests that such aberrant flow also relates to angiogenic responses in in vivo situations of vascular malformations.

High flow lesions are the arteriovenous type of malformations, which are often treated by means of embolization of the feeding arteries. Many of the embolized AVM showed an angiogenic response, which is not surprising since the ischemic tissue damage induced by the embolic vascular occlusion is a powerful inducer of angiogenesis. And also inflammation, whether or not related to embolization, could elicit in itself an angiogenic response. Interestingly, a number of non-embolized high flow lesions also showed a similar angiogenic response, implicating that the intrinsic flow forces of the lesions may induce angiogenesis. This is also supported by our finding of endothelial leakage of von Willebrand factor, which can occur as a direct effect of flow related changes such as shear stress.[19]

Irrespective of the underlying causes of microvascular growth in high flow AVMs, these observations could lead to novel treatment strategies in the management of mass forming complications in AVMs, such as anti-angiogenic agents. Such drugs, for example interferon alfa, vincristine and topical imiquimod[20], could be applied when the presence of a microvascular response is proven by means of a biopsy. Anti-angiogenic agents are also used in the treatment of other vascular lesions in their proliferative phase, such as infantile hemangiomas and pyogenic granulomas. We established in our studies that the angiogenic response in AVM follows the same course of proliferation and maturation as is seen these lesions that are presently treated with anti-angiogenic therapy, albeit with variable success.

Recently propanolol, which acts as a bFGF and VEGF inhibitor, was added as treatment for complex infantile hemangiomas.[21,22] This seems to be very effective, with promising results and minor side-effects. We suppose that a similar approach can be also effective in the management of (embolized) high flow AVMs.

Part III. Vascular malformations at specific topographic sites

Chapter 8. Hypertrophy in facial capillary malformations: clinical and pathological findings in 11 patients.

Capillary malformations are flat well demarcated reddish lesions that grow proportionally and never regress. With increasing age the lesions can thicken and darken in color, and also cobblestone appearances have been described.[23] In our clinical series we identified 11 patients with generalized hypertrophy of the lip against the background of a facial capillary malformation. Since this finding is highly unusual for capillary malformations we performed detailed histopathological analysis of the lesions. All surgical specimens showed dilated thin walled microvessels in the superficial dermis without a neural component, consistent with the capillary malformation. The hypertrophic areas were composed of large multinodular conglomerates of thick walled vessels with a substantial increase in nerve fibers, consistent with a diagnosis of a venous malformation. As usually occurs in this type of malformations the veins extended deep into the facial musculature. In contrast with the depletion of a neural component in the upper part of the lesion we found a remarkable increase in nerve fibers adjacent to the thick walled veins.

Not all cases with vascular malformations are easy to classify, which is illustrated by these 11 patients with generalized hypertrophy of the lip in combination with a capillary malformation. The generalized hypertrophy in these patients with a capillary malformation is caused by an underlying venous malformation, and thus should be considered as capillary-venous malformation. This finding has important clinical and treatment implications, because these two different types of malformations need different management strategies. It is of interest whether an underlying genetic mutation is involved in the pathogenesis of this combined capillary-venous malformation.

Chapter 9. Two cases of cardiac arteriovenous malformations complicated by a local angioproliferative process.

Vascular malformations can occur in other parts of the body than in skin and soft tissue. Vascular malformations of the heart are extremely rare with only a few cases of the arteriovenous type of vascular malformation (AVM) reported. We investigated the pathology of two additional cases, of which one witnessed sudden cardiac death and the other died due to untreatable heart failure. Histopathological analysis of the heart combined with immunohistochemistry revealed in both cases a similar microvascular proliferation as we noticed in our series of AVMs in soft tissue and skin. One of the two patients was known since birth with an asymmetrical hypertrophy of the ventricular septum, diagnosed as hypertrophic cardiomyopathy (HCM).

The cases show that a microvascular proliferative response in the setting of an AVM is not unique for occurrence in skin and/or soft tissue but can also manifest in internal organs, like the described cases located in the heart. Others have reported similar cases in the gut,[24] also leading to severe symptomatology. The cases clearly illustrate the danger of an extensive vasoproliferative response when it is located in vital organs, while both cases showed a fatal course. We suppose that in our two cardiac cases a hypoxic environment in the myocardium of the progressively failing heart served as a substrate for the onset of angiogenesis.

Chapter 10. Congenital vascular malformations - cerebral lesions differ from extracranial lesions by their immune expression of the glucose transporter protein GLUT1.

Cerebrovascular malformations were investigated for the presence of the glucose transporter protein GLUT1, which is normally expressed in endothelial cells of the pre-existing microvasculature of the brain and absent in the vasculature of the choroid plexus and extracranial vasculature without a barrier function.[25] From extracranial arteriovenous malformations (AVMs) is known that they show an absence of GLUT1 expression which distinguishes them from infantile hemangiomas of skin and soft tissue.[6] Four cerebral AVMs, including one choroid plexus AVM, 3 cerebral cavernous malformations (CCMs) and 3 extracranial facial AVMs were immunostained with anti-CD31 and anti-GLUT1 in a double staining procedure which was further analyzed with the use of spectral analysis software. All 7 cases of cerebrovascular malformations showed co-localization of GLUT1/CD31 of endothelial cells of the vessels in the malformation. There was no expression of GLUT1 in the extracranial AVMs.

In contrast to extracranial vascular malformations GLUT1 cannot be used in the differential diagnosis between hemangiomas and vascular malformations, while GLUT1 is expressed in the pre-existing microvasculature of the brain. However the expression of GLUT1 in cerebrovascular malformations, including the one located in the choroid plexus, suggests that the endothelial phenotype of the local vascular beds from which they are derived during embryogenesis is retained in vascular malformations.

Part IV Nerves in vascular malformations

Chapter 11. Presence of a distinct neural component in congenital vascular malformations relates to the histological type and location of the lesion.

In this study the presence of an intralesional component of nerve bundles in vascular malformations was investigated in 130 resection specimens (83 AVM, 33 VM and 14 LVM). All sections were immunostained with anti-S100 antibody to screen for the presence and extent of intralesional mature nerves bundles. Of all cases, 74% showed a substantial increase of intralesional nerves in close opposition to the vessels of the malformation. The nervous component appeared to be more extensive in the head and neck area and upper extremities than in other topographic sites. Most cases with an increase in nerve elements were of the arteriovenous type (87% of all AVM), whereas 55% of the venous malformations had an increase in nerve density, and lymphatic malformations showed a complete absence of nerves.

Congruency of the development of peripheral nerves and blood vessels has been well described and usually nerves and blood vessels follow each other during development.[26,27] In line with this concept we were interested to see whether vascular malformations, considered as being developmental anomalies, also contain a substantial neural component. In this chapter we have proven that this is indeed the case. The abundant presence of intralesional mature nerves in the majority of congenital vascular malformations suggests that, at least in a large subset of lesions, neural components are an integral part of the developmental disorder. It was already known from previous studies that vascular malformations can also contain an excess of other mature tissue components, such as adipose, hyalinized or myxoid tissue.[28] This is known from deep intramuscular malformations, in which the amounts of adipose tissue are so abundant that they can be mistaken for intramuscular lipoma (Chapter 2, Figure 8).

The clinical implications of these nerve profiles in venous malformations are discussed in **Chapter 12. Nerves in congenital vascular malformations - a painful association?** In this chapter we give a reply on the study by Gokani and colleagues, who histopathologically evaluated the nerve profiles in 29 surgically resected congenital vascular malformations, with the same techniques as we used. Not surprisingly most of their findings were similar to ours, such as increase in nerves in several types of malformations. The authors believe that the observed aberrant nerve profiles in venous malformations cannot be held responsible for discomfort and pain which is according to them a symptom in >90% of patients with venous malformations, since in their studies also arteriovenous malformations and capillary malformations showed a similar intralesional increase in nerves. To our opinion such a statement is arguable, since pain can be a serious symptom also in cases of AVMs, especially

in the craniofacial region, and an increase in nerves in capillary malformations is in disagreement with the literature[29] and also with our findings (Chapter 8). We do agree that complications such as thrombosis, formation of phleboliths, hemorrhage, and chronic inflammation can lead to clinical discomfort including pain, but this does not rule out the possibility that local nerve increase could at least aggravate such symptoms.

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