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Vasculogenesis in Infantile Hemangioma

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Abstract

Infantile hemangioma is a vascular tumor that occurs in 5-10% of infants of European descent. A defining feature of infantile hemangioma is its dramatic growth and development into a disorganized mass of blood vessels. Subsequently, a slow spontaneous involution begins around one year of age and continues for four to six years. The growth and involution of infantile hemangioma is very different from other vascular tumors and vascular malformations, which do not regress and can occur at any time during childhood or adult life. Much has been learned from careful study of the tissue morphology and gene expression patterns during the life-cycle of hemangioma. Tissue explants and tumor-derived cell populations have provided further insight to unravel the cellular and molecular basis of infantile hemangioma. A multipotent progenitor cell capable of de novo blood vessel formation has been isolated from infantile hemangioma, which suggests that this common tumor of infancy, long considered to be a model for pathologic angiogenesis, may also represent pathologic vasculogenesis. Whether viewed as angiogenesis or vasculogenesis, infantile hemangioma represents a vascular perturbation during a critical period of post-natal growth, and as such provides a unique opportunity to decipher mechanisms of human vascular development.

Keywords

angiogenesis; endothelial cells; endothelial progenitor cells hemangioma; vasculogenesis; VEGF-receptors

Introduction

Infantile hemangioma (IH) is a benign vascular neoplasm of infancy that appears soon after birth in 5-10% of children of mixed European descent (1-3). It occurs more often in females compared to males and is also more prevalent in premature infants. Most IH remain relatively small and pose no serious threat or complication to the baby. However, a small number of IH grow so large they lead to tissue and organ damage and in some cases become life-threatening. Examples of two lesions, one small and one of moderate size, in two different infants at six months of age are shown in Figure 1.

Depending on the size and location of the hemangioma, many serious problems can ensue. For example, eyelid IH can cause deprivation amblyopia, a subglottic IH can compromise the airway, and extremely large IH can cause high-output congestive heart failure (4). Some IH ulcerate and bleed, which then requires transfusion and/or surgery. Infants with large facial hemangiomas are at risk for cerebral infarction (5). Ectopic expression of type 3 iodothyronine deiodinase in IH has been shown to inactivate thyroid hormone systemically and cause

hypothyroidism (6). Finally, 40-80% of IH leave permanent cutaneous residua after tumor involution, which can be particularly disfiguring in children with facial IH.

Current treatments for children with endangering IH are limited, and include primarily corticosteroids(7), which have many adverse effects. Furthermore, approximately 30% of hemangiomas do not respond to corticosteroids, prompting active investigations for new treatments. Vincristine, a chemotherapeutic agent, has emerged as a second line treatment for IH that do not respond to steroids (8,9). Interferon- α was used for severe, problematic hemangiomas (10,11) but irreversible neurologic toxicity has been associated with interferon- α administration to infants with IH (12,13), and therefore it is now rarely used. A recent report has shown the β -adrenergic receptor antagonist propranolol to be effective against severe IH (14). However, the mechanism of action and potential for adverse effects in infants is currently unknown due to the serendipitous nature of this discovery. Therefore, even with the encouraging new results with propranolol and the widely-accepted use of steroids, there is still a pressing need for safe and fast-acting treatments for infants with endangering IH.

Life cycle of IH

IH displays a unique life-cycle of proliferation followed by involution. The “proliferating phase” spans the first several months of infancy, with most of the growth occurring by five months of age (15). Histological analyses of IH tissue sections show that the tumors are highly cellular, with clusters of plump cells expressing endothelial markers and small vascular channels with barely discernable vessel lumens (Figure 2). Among the vascular channels there are proliferating “interstitial” cells but little connective tissue (16,17). The human stem/progenitor cell marker CD133 can be detected on sparsely scattered subsets of cells (Figure 2G), indicating the undifferentiated status of cells in the proliferating phase. The expression of angiogenic factors and receptors, including vascular endothelial growth factor (VEGF)-A (18,19), VEGF-receptors VEGFR-1 and VEGFR-2, Tie2 and angiopoietin-2, shown by immunohistochemistry and in situ hybridization techniques, supports the notion of nascent endothelia in the proliferating phase (16,20-22).

Growth of IH slows dramatically in the “involuting phase”, which typically begins soon after the child's first birthday and lasts for four to six years. As involution proceeds, vascular channels become more prominent and are lined with flattened endothelial cells (Figure 2). The clusters of plump immature cells are no longer evident. There are fewer interstitial cells and deposition of extracellular matrix occurs, with multi-laminated basement membranes building up around the enlarged vessels. Pericytes, shown by expression of the marker NG2, neural/glial antigen-2 (Figure 2H), are embedded within the basement membranes and surround the hemangioma vessels. Hence, it appears that the involuting phase of IH coincides with the de novo formation of mature blood vessels (Figure 2).

The endothelium lining these vascular channels has been extensively characterized by immunohistochemical staining and shown to bear similarities with placental endothelium (23,24), which has brought forth much discussion and debate about whether or not IH arises from a displacement of placental cells during fetal development (25,26). Two molecular genetic studies have determined that hemangioma-derived cells originate from the child and not the mother (27,28), shedding some insight on the origin of the tumor.

A hallmark of IH is that eventually, the disorganized mass of vessels will regress when the child reaches eight to ten years of age. This final, “involved phase” is characterized by sparse, thin-walled vessels, around which the characteristic multi-laminated basement membranes remain, with large feeding and draining vessels evident as well. However, the bulk of involuted hemangioma tissue is composed of adipocytes and connective tissue (Figure 2). For some hemangiomas, the mass of fat that replaces the proliferating IH can be of a similar size. This

raises intriguing questions on the cellular origin and source of the adipocytes in this final phase of IH.

Angiogenesis or Vasculogenesis?

IH has long been considered an angiogenic disease because of the tangled disorganized mass of blood vessels in the tumor (18,29). The detection of angiogenic factors such as basic fibroblast growth factor (bFGF) and VEGF-A within the tumor have supported this concept (18,19). However, an alternative is that IH arises by a process more akin to vasculogenesis, i.e., the de novo formation of vessels from progenitor cells. This concept is consistent with the long-noted presence of immature cells in IH. The immature cells could arise from stem/progenitor cells that reside within the lesion, for example the dormant angioblasts proposed by Pack and Miller(30) or from cells recruited to the IH from a reservoir of stem/progenitor cells such as the placenta or bone marrow. Once tumor growth has been initiated, angiogenesis may also contribute, with in-growth of vessels from the surrounding tissue. Our view is that understanding the precise cellular mechanisms of blood vessel formation in IH will be a catalyst for the discovery of new drugs and strategies for the treatment of children with suffering from endangering or life-threatening IH.

CELLULAR COMPONENTS OF IH

The idea that IH arises from an undifferentiated stem/progenitor was first suggested in the 19th century and further developed in the mid 20th century as IH was described as sequestered embryonic mesoderm or activation of dormant angioblasts (30-33). In the 1990s, Smoller and Apfelberg speculated that IH arises from a primitive vascular progenitor that gives rise to endothelial cells and pericytes(17). An important approach to compliment histological studies has been to dissect the lesions into purified cell types that can be studied, tested and compared in the laboratory. Here in we will discuss several papers that have made important inroads in understanding the cellular complexity of hemangioma.

Immature endothelial cells

A pioneering study by Mulliken and colleagues showed that it was possible to isolate hemangioma-derived endothelial cells (HemECs) and study their phenotype in vitro (34). Dosanjh and colleagues took this a step further and compared the in vitro characteristics of HemECs to fetal skin endothelial cells (isolated from second trimester pregnancy terminations) and neonatal skin endothelial cells (isolated from newborn foreskin following routine circumcision) (35). The HemEC and fetal ECs exhibited a spindle-shaped morphology when grown in vitro and produced type I collagen but not type IV collagen. Conversely, neonatal ECs formed a cobblestone-like monolayer and secreted type IV collagen but not type I collagen. Notably, the HemECs and fetal ECs exhibited a diffuse intracellular pattern of CD31, also known as platelet endothelial adhesion molecule-1 (PECAM-1), and vWF immunostaining, which suggested these were not fully differentiated endothelial cells. The authors concluded their study with the speculation that IH may represent a dysregulated endothelial differentiation and maturation (35), a concept that has now been extended by many laboratories including ours (21,36,37). The dysregulation could cause a delay in endothelial differentiation and result in an ill-timed and disorderly vascular proliferation, as seen in proliferating IH.

We isolated hemangioma-derived endothelial cells (HemECs) from ten different proliferating IH specimens, and found these cell populations displayed many features of human ECs, for example expression of endothelial markers. Together with our collaborators in the Olsen laboratory, we showed the HemECs were clonal and exhibited increased growth and migratory properties (36). The clonality and altered properties of the HemECs strongly suggested an intrinsic defect in these cells, perhaps caused by a somatic mutation which would lead to clonal

expansion. Clonality was also shown in cells analyzed directly from tissue sections of proliferating IH by Marchuk and colleagues (38), which argued against the clonality being due to in vitro culture favoring growth of a small number of cells. Marchuk's finding of clonality in a wide swath of unselected cells recovered from tissue sections suggests that the entire tumor might be derived from a single progenitor cell.

Endothelial progenitor cells

We subsequently set out to isolate potential precursors of HemECs from proliferating IH by selecting cells that co-expressed the human stem cell marker CD133 and an endothelial marker; we refer to these as hemangioma-derived endothelial progenitor cells (HemEPCs) (22,39). To test whether or not the HemEPCs give rise to HemECs, we examined cellular growth, migration and adhesion of HemEPCs and HemEC in parallel, with the hypothesis that the cells would exhibit the same properties, in particular the HemEPCs would show the same stimulatory response to endostatin (an angiogenesis inhibitor) (40) that we found in HemECs (36). The HemEPCs were stimulated by endostatin, but unexpectedly so were normal healthy cord blood-derived EPCs (39). In summary, HemEPCs, HemECs and cord blood EPCs are similar to each other in several in vitro assays including mRNA transcriptional profiling for expression of cell-cell and cell-matrix adhesion molecules. Consistent with the study of Dosanjh and colleagues (35), HemECs, as well as HemEPCs and cord blood EPCs, could be distinguished from neonatal human dermal microvascular endothelial cells in our in vitro assays. Our conclusion is that HemEPCs and HemECs are immature endothelial cells and share properties with cord blood EPCs. This raises the question of whether circulating EPCs are recruited into proliferating IH lesions and if so, do they contribute to growth of the tumor. In support of this, Kleinman and colleagues reported an increased number of circulating CD133+/CD34+ EPCs in children with hemangioma(41). However, the age-matched controls were somewhat older (average age 38 months) than the hemangioma patients (average age 25 months). Since levels of circulating EPCs are likely to vary during infancy and early childhood, it would be important to extend these studies to a larger number of more closely matched controls to verify the extent to which circulating EPCs are elevated in IH.

Interstitial cells

Although IH is noted for its vascularity and angiogenic profile (18), some investigators have focused on the cells located between the vascular channels(17). Smoller and Apfelberg described these cells as "interstitial tumor cells," a heterogeneous mix of cells with both cuboidal and spindle-shaped morphology. The interstitial cells did not express von Willebrand factor (vWF), a marker of differentiated endothelial cells, but instead most of them were positive for factor XIIIa, a marker of dermal dendrocytes, some were positive for CD34, a stem cell and endothelial marker, and even fewer were positive for α -smooth muscle actin (α -SMA), a pericyte/smooth muscle cell/myofibroblast marker. Importantly, most of the proliferating cells in the IH samples were located in the interstitial, non-vascular areas. Based on their analyses, the authors put forth the concept that proliferating phase IH is composed of a primitive progenitor cell capable of differentiating into endothelial cells and pericytes(17). This concept was extended in a subsequent study that showed increased expression of the anti-apoptotic gene *bcl-2* in the interstitial cells, suggesting these cells might have a survival advantage (16). Intriguingly, their quantitative analyses suggested that proliferation and *bcl-2* expression in the interstitial cells was prolonged in lesions with markedly more vascular channels. A more in-depth understanding of this observation might provide a means to predict which IH will grow to an endangering size.

Pericytes

Pericytes, sometimes called mural cells, surround the newly formed vessels in IH, however to date there are no reports of isolation and/or culture of pericytes from hemangioma tissue. Immunostaining shows nerve/glial antigen-2 (NG2), a proteoglycan and marker of pericytes (42,43), prominently expressed in the perivascular cells in a proliferating IH tissue section (Figure 2G). Others have shown that the perivascular cells in IH are α -SMA- and CD146-positive cells (44). Pericytes in several human tissues have been shown to be CD146+/NG2+/PDGFR- β + with absence of endothelial, hematopoietic and myogenic cell markers. Furthermore pericytes exhibit many of the same properties as mesenchymal stem cells, including osteogenic, chondrogenic and adipogenic differentiation potential (45). Recently, a subset of perivascular cells aligning the vasculature of white adipose tissue were shown to be adipogenic progenitor cells (46). Therefore, one might speculate that the pericytes in IH are also capable of multi-lineage differentiation potential, and specifically adipogenesis, which would provide a potential for the adipocytes that appear in the involuted phase (Figure 3).

Multipotent stem cells

We recently reported on a multipotent progenitor-like cell isolated from over thirty different proliferating IH specimens – we refer to the cells as hemangioma-derived stem cells (HemSCs) (47). The HemSCs exhibit robust proliferative and clonogenic capability, and can differentiate into cells of multiple lineages, thereby fulfilling two important features of stem cells. HemSCs express VEGFR-1/Flt-1, neuropilin-1 (NRP-1), but do not express CD31/PECAM-1 or VE-cadherin, two reliable endothelial markers. Furthermore, the HemSCs express CD90, a mesenchymal cell marker, which indicates the HemSC are phenotypically more similar to mesenchymal cells than to endothelial cells. It is of interest to note that CD90 was the gene most differentially up-regulated in proliferating compared to involuting phase IH in a micro-array analysis in IH (48).

Clonal HemSCs, expanded *in vitro* from single cells, produce human GLUT-1-positive microvessels *in vivo* seven to fourteen days after sub-cutaneous implantation into immunodeficient mice (49). GLUT-1 is an immuno-diagnostic marker for IH (50), and thus its presence indicates the vessels express an important marker of the IH phenotype. HemSCs that had differentiated into endothelium *in vivo*, as determined by cell surface CD31 expression, could be retrieved and implanted into secondary recipients, where the cells formed blood vessels once more. This vasculogenic potential is restricted to the HemSC because HemEC, HemEPCs, cord blood EPCs, normal human fibroblasts and bone marrow-derived mesenchymal stem cells do not form vessels in this *in vivo* model. Over time, the human blood vessels formed from HemSCs diminished and adipocytes became evident, reminiscent of the involuted phase of IH. GFP-labeled HemSCs were shown to form the adipocytes, in addition to the endothelial lining of the vessels (Figure 3A), providing further confirmation that the vessels and adipocytes were not murine-derived (49). The cellular origin of the NG2+ perivascular cells in IH has not been shown but we speculate that HemSC may have the potential to differentiate into pericytes (Figure 3A). Based on the ability of adipose perivascular cells to differentiate into fat (46), we propose an alternative pathway in which HemSC give rise to pericytes in the proliferating and early involuting phases of IH, which then in turn differentiate into adipocytes (Figure 3B). Further studies are required to elucidate the cellular origin and fates of the pericytes in IH.

In summary, our findings provide evidence for a stem cell origin of hemangioma, which is in contrast to the long-held view that hemangioma arises from endothelial cells and angiogenesis (29). To our knowledge, the HemSC is the only post-natal, non-bone marrow-derived cell that solely, on its own, can form functional vascular networks *in vivo*. Our work, and that of others, has shown that rapid and substantial vascular network formation from normal human

endothelial cells requires a mesenchymal support cell (51-53). Thus, the HemSC is truly a unique cell in its ability to form vessels in vivo when implanted alone without a mesenchymal support cell. Whether IH represents an environmental perturbation of a normal post-natal stem cell or the HemSCs harbor genetic alteration(s) that contribute to vasculogenic and adipogenic activity must be determined in future studies.

Other cellular components

IH contains many additional cellular components, which have been primarily studied by histological analyses of tissue sections. Mast cells have been noted in all three phases of IH, with the highest level appearing in the early involuting phase(54). It has been suggested that mast cells signal the beginning of involution (55), however to date the precise role of mast cells in IH has not been determined. Myeloid cells have also been detected in proliferating phase IH and suggested to function in a pro-angiogenic manner (56). The potential contribution of cells from the bone marrow was also suggested by Nguyen and colleagues (57) based on detection of cells expressing HLA-DR and CD68 antigen in a close proximity to vessel structures in hemangioma tissue sections. The cells were shown to be distinct from pericytes or immune cells such as T-cells, B-cells, NK cells or mast cells. Their morphology and immunophenotype was suggested to be most closely aligned with antigen presenting cells of the monocyte/macrophage/dendritic lineage. Based on a hypothesis that the angiogenic vessels in proliferating IH may secrete soluble factors that induce sensory nerve fibers growth, and in turn that neuropeptides may stimulate IH endothelial growth, Jang and colleagues looked at calcitonin gene-related peptide (CGRP) and protein gene product 9.5 (PGP 9.5) in IH (58). They found dramatically increased numbers of CGRP+ nerve fibers in proliferating IH compared to involuting IH, and suggested that the hemangioma vessels and nerve fibers may regulate each other. In summary, the inter-relationships among these various cell types and their functions in IH must be explored to fully understand the vasculogenic mechanisms that contribute to the pathogenesis of hemangioma.

MOLECULAR PATHWAYS IN IH

VEGF/VEGFRs

Many investigators have obtained clues and probed pathways to expand our nascent understanding of the vasculogenesis and angiogenesis in IH. In vivo studies have consisted of expression analyses on surgically-removed IH tumor specimens while in vitro studies have benefited from use of explants and purified hemangioma-derived cell populations. VEGF-A has understandably been a major focus because of its enormous importance in so many aspects of vascular development and pathologic angiogenesis. Early studies showed VEGF-A protein in the vessels of proliferating IH (18,19,59), and more recently VEGF-A was shown to be increased HemECs from three different IH specimens (60). Other studies did not reveal significant expression of VEGF-A transcripts in proliferating IH (48) or in cultured HemECs (21). However, VEGF secretion by stromal cells isolated from IH has been reported (59). Perhaps the VEGF-A protein detected on hemangioma vessels by immunostaining was due to VEGF secretion from the stromal cells and localization on endothelial cells via VEGF-receptors. The stromal cells isolated by Berard and colleagues do not react with antibodies against endothelial markers such as vWF and Ulex europeus agglutinin (UEA-1), but express α -SMA, VEGF-A and the VEGF-A receptor KDR, also known as VEGFR-2. The authors did not speculate on the ability of the stromal cells to become the endothelial cells in the tumor, but they shed light on an intriguing cellular component of IH that may be related to the HemSC reported by our laboratory (49).

The relevance of VEGF-A in the pathogenesis of IH was also demonstrated in a study in which VEGF-A levels in patient serum were found to be significantly higher in proliferating compared

to involuting hemangioma. Interestingly, after steroid therapy, VEGF-A levels were reduced compared to pre-treatment (n=6) (61), suggesting a putative mechanism of action of steroids in IH.

The role of VEGF-A has been further explored by real-time quantitative PCR for VEGF-receptors in the tissue samples of proliferating, involuting and involuted hemangioma (20). Expression levels of VEGFR-1/Flt-1, VEGFR-2/KDR, and NRP-1, and neuropilin-2 (NRP-2) were measured and normalized to GAPDH and also normalized to VEGFR-2/KDR to account for endothelial content of the IH specimen. RNA from human neonatal foreskin and placenta were used for comparisons. VEGFR-1/Flt-1 was found to be consistently under-expressed in seven different IH specimens compared to foreskin, placenta and also 7 different congenital hemangioma specimens (20) whereas the other VEGF-receptors were not. Decreased VEGFR-1/Flt-1 expression was also reported in a separate study (60). During development, VEGFR-1^{-/-} mice die at E8.5 due to disorganized blood vessels and endothelial cell over growth (62) – two features somewhat reminiscent of IH. Therefore, it is possible that diminished or defective VEGFR-1 signaling is part of the pathogenesis of IH.

The specific IH-derived cell type in which VEGFR-1 is down-regulated may be the HemEC. Decreased VEGFR-1 protein and mRNA, measured by ELISA and real-time quantitative PCR respectively, was shown in several hemECs compared to different types of cultured human endothelial cells, e.g., human umbilical vein endothelial cells (HUVECs) and human dermal microvascular endothelial cells (HDMECs), cultured under the same conditions (60). The reduced expression of VEGFR-1, which acts as a decoy receptor in some settings, could result in increased VEGF-A binding to VEGFR-2 (Figure 4). Evidence to support this was shown by increased, constitutive levels of phosphorylated VEGFR-2 and its target ERK in HemECs and increased proliferation of HemECs (60). The presence of a potential binding site for transcription factor NFAT (nuclear factor in activated T cells) in the promoter region of flt-1, and the fact that NFATc1 overexpression stimulated VEGFR-1 expression suggests that attenuated NFAT activity is responsible for low VEGFR-1 expression in HemECs(60). Surprisingly NFAT transcript levels in hemECs were comparable to HDMECs, but NFAT-regulated genes as Down syndrome critical region protein-1 (also known as Regulator of Calcineurin-1, RCAN1) and cyclooxygenase-2 (PTGS2) were downregulated in hemECs indicating that the defect involves NFAT activity and not expression. Jinnin and colleagues went on to show that NFAT activity was disrupted in HemECs due to mutations in VEGFR-2 and TEM8 that affect complex formation with β 1-integrin (Figure 4). The mutations will be discussed in depth below.

Genetic alterations

Most hemangiomas occur sporadically but the potential contribution of familial and/or somatic mutations to IH has been studied in several laboratories. Blei and colleagues postulated a familial form of IH in a study of five families with multiple generations affected by IH, with evidence of an autosomal dominant inheritance (63). A subsequent linkage analysis conducted in three of these families established a genetic linkage with chromosome 5q (64). The region, 5q31-33, contains three candidate genes involved in blood vessel growth: FGFR4 (Fibroblast Growth Factor Receptor 4), PDGFR- β (Platelet Derived Growth Factor Receptor) and FLT4 (Fms-related tyrosine kinase-4). The existence of somatic mutational events in chromosome 5q has been explored with micro-satellite marker analysis. In micro-dissected tissue from non-familial IH specimens, loss of heterozygosity (LOH) was detected in chr5q suggesting an association with sporadic, non-familial IH (38). A mutation was reported very recently in the dual specific phosphatase-5 (dusp-5), localized on chr 10q25, in one out of three IH specimens (65). Dusp-5 was discovered as a vascular specific gene, and shown to be involved in the dephosphorylation of MAPK (66,67). The reported mutation is a T→C substitution resulting

in a change from serine to proline, rendering the phosphatase unstable. The authors found the same mutation in 16/21 vascular malformations indicating a role for Dusp-5 in the vascular development.

Because of the accumulated evidence suggesting IH can be familial, in addition to the more common sporadic forms, and in light of NFAT-VEGFR-1 dysfunctions, the Olsen laboratory sequenced 24 genes involved in EC proliferation, migration and adhesion to extracellular matrix, or in regulation of VEGF-A expression. From this effort, germline “risk-factor” mutations were identified in the integrin-like molecule TEM-8 (tumor endothelial marker-8) and in VEGFR-2 (60). TEM8 is selectively expressed in tumor blood vessels; its N-terminal domain encodes an anthrax toxin receptor (ATR) (68). TEM8 plays a positive role in angiogenesis and is over-expressed in HUVECs during the initiation of tube formation in vitro. The TEM8 mutation identified by Jinnin and colleagues is a G→A transition that replaces alanine with threonine in the transmembrane domain. Introduction of this mutant TEM8 into HDMECs decreased VEGFR-1 expression and increased VEGFR-2 and ERK phosphorylation, indicating the mutant TEM8 acts in a dominant-negative fashion. The VEGFR-2 mutation is a T→C transition that replaces cysteine with arginine in the extracellular domain. When this mutant VEGFR-2 was overexpressed in HDMECs, the decrease in VEGFR-1 was limited, whereas over-expression of wild type VEGFR-2 in HemECs bearing the mutant VEGFR-2 restored VEGFR-1 levels to normal and decreased phosphorylated-VEGFR-2 and phosphorylated-ERK. These results indicate the Cys to Arg substitution in VEGFR-2 is a loss-of-function mutation. In summary, the effects of these germline, “risk-factor” mutations in endothelial cell-based assays led to the model depicted in Figure 4. The mutant VEGFR-2 and TEM8 can sequester β 1-integrin into a protein complex that negatively regulates β 1-integrin activity and NFAT transcriptional function, resulting in decreased expression of VEGFR-1. The protein complex was identified in HDMECs as well as in HemECs but it is more abundant in HemECs because the mutant TEM8 and VEGFR-2 form the complex with higher affinity resulting in NFAT activity suppression. Lower expression of the decoy VEGFR-1 in hemECs modulates the availability of VEGF-A (Figure 4B). VEGF-A is more available to bind to VEGFR-2 thereby causing a constitutive activation of VEGFR-2, elevated levels of phospho-ERK, and ultimately resulting in increased proliferation of HemEC compared to normal human endothelial cells. Interestingly, strong expression of phosphorylated MAPK has been shown in benign endothelial tumors such as capillary hemangioma and pyogenic granuloma (69). In summary, these studies represent the most in-depth elucidation of a molecular pathway in the pathogenesis of IH. This new mechanism provides a strong incentive to test anti-VEGF-A agents or VEGFR-2 and MAP-kinase inhibitors to treat severe cases of IH.

Other pathways

Another receptor-ligand system involved in normal and pathogenic angiogenesis is Tie2-angiopoietins. Increased Tie2 mRNA and protein have been shown in cultured HemECs, with a corresponding enhanced response to angiopoietin-1 (Ang1). Both Ang1 and angiopoietin-2 (Ang2) induce phosphorylation of Tie2 and are required for viability in mice (70,71). Both polypeptides promote angiogenesis in presence of VEGF-A in vitro but in vivo they have opposite effects. Overexpression of Ang1 in tumor cells leads to increased vessel maturation and decreased tumor growth (72,73). Ang2 is antagonist of Ang1 for Tie2 and acts as a survival factor for ECs through PI3K/AKT pathway. In a model of diabetic retinopathy overexpression of Ang2 resulted in enhanced vascularization and impaired pericytes recruitment (74). As is the case for normal human ECs, Ang2 is expressed by HemECs and appears to be more abundantly expressed in IH tissue compared to Ang1 (21). However, at present, the role of Tie2 and Ang2 in the abnormal growth and regression of IH blood vessels remains to be determined. Related studies in a murine tumor model have shown that blocking Ang2 function

with soluble Tie2 receptor or down-regulating Ang2 pharmacologically inhibited growth of a murine endothelial tumor cell line (bEnd.3 cells) (75). The mechanism of action proposed involves Src-induced activation of cellular tyrosine kinase signaling pathways, but there are no parallel studies showing such a pathway is operative in IH or in IH-derived cells.

Insight into the pathogenesis of IH may also be gained from transgenic murine models. For example, sustained AKT activation has been shown to cause vascular malformations and therefore may also be involved in IH (76). For example, sustained endothelial expression of myrAKT1 in transgenic mice results in abnormal blood vessels, with structural anomalies similar to tumor blood vessels (77). The mTOR inhibitor rapamycin inhibited the endothelial myrAKT1 and reversed the abnormal vascular morphology. Since both the VEGFR-2/VEGF-A and Tie2/Ang2 receptor/ligand systems can stimulate AKT signaling in endothelial cells, there may be a role for AKT in IH. Furthermore constitutive and VEGF-A induced AKT phosphorylation has been detected in hemECs at a higher level compared to HDMECs (E. Boscolo, unpublished data).

Hypoxia may also play a role in IH, particularly in the early proliferating phase when only small nascent vessels are present within the tumor. Kleinman and colleagues analyzed HIF-1 α expression and distribution in IH. HIF-1 α stabilization is increased in proliferating phase IH compared to involuting phase IH. The authors propose HIF-1 α induces production of VEGF-A, MMP9 and SDF-1 α , which in turn induces mobilization and homing of EPC during post-natal vasculogenesis responsible for hemangioma growth (78).

Many other factors have been detected in IH and suggested to play a role but mechanistic studies have not yet been reported. In proliferating IH, MCP-1 (monocytes chemoattractant protein-1) is highly expressed by the α -SMA-positive perivascular cells and by macrophages, localized in proximity to proliferating endothelial cells (79). MCP-1 is involved in initiating and maintaining recruitment of angiogenic macrophages suggesting the possibility that MCP-1-recruited macrophages play a role in IH. Interestingly, after treatment with dexamethasone or interferon- α , levels of MCP-1 decreased as well as macrophage recruitment and activation (79). Expression of ICAM-3 (intercellular adhesion molecule-3) has been detected by immunohistochemistry in hemangioma tissue. ICAM-3 is not expressed in ECs from normal tissues but it is expressed in benign and malignant tumors. Interestingly, ICAM-3 expression in hemangioma is localized in immature areas and is lower in the proximity of mature, well defined vessels. Another cell adhesion molecule, E-selectin, was detected by immunohistochemistry in proliferating hemangioma and shown to be co-localized with CD31 and Ki67 in proliferating endothelial cells (80). E-selectin has been implicated in angiogenesis and also homing of endothelial progenitor cells to ischemic muscle (81), homing of T cells to inflamed skin (82), and of human CD34+ progenitors to bone marrow (83). This raises the intriguing possibility that E-selectin may be involved in homing of cells into proliferating IH lesions.

Conclusion

IH is a common tumor of infancy best known for its explosive growth of abnormal blood vessels. The cellular and molecular mechanisms that contribute to its growth and involution have eluded our full understanding until recently. The identification of multipotent stem cells that give rise to hemangioma blood vessels and adipogenesis in vivo provides an appropriate cellular context for elucidating molecular mechanisms and new therapies. VEGF-receptor signaling, coordinated presentation of cell surface multiprotein complexes and transcriptional regulation are novel and exciting targets for intervention and will also shed important insight on critical control points in human vascular development.

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Figure 1. Two examples of infantile hemangioma

In panel A, one prominent lesion and two smaller lesions are seen. Multiple hemangiomas, as seen here, occur in 20% of cases. In panel B, a facial hemangioma with potential to affect the eye is shown.

Figure 2. Histology and cellular phenotyping in proliferating, involuting and involuted phases of IH

Panels A-C, hematoxylin and eosin staining; Panels D-F, immunostaining for human CD31, an endothelial marker; Panel G, immunostaining for CD133, a human stem and progenitor cell marker CD133; Panel H, immunostaining for NG-2, a pericyte marker; Panel I, immunostaining for perilipin-A, an adipocyte marker that prominently encircles the lipid storage droplets in adipocytes. Scale bars are 100 μm. Panels G and H contributed by Arnaud Picard, M.D.

Figure 3. Multipotent hemangioma-derived stem cells (HemSC) differentiate in vivo and in vitro into endothelium and adipocytes

The cellular origin of pericytes in IH has not been established, as indicated by the dashed line in panels A and B. In panel B, we speculate that the pericytes surrounding the IH blood vessels may differentiate into adipocytes in the involuted phase.

Figure 4. VEGFR-1 regulation in endothelial cells

Panel A, when β_1 -integrin in endothelial cells is activated, it promotes NFAT translocation into the nucleus where it binds to the VEGFR-1 promoter to stimulate VEGFR-1 transcription. β_1 -integrin-induced NFAT activation is inhibited when a complex with VEGFR-2 and TEM8 is formed. Mutations in TEM8 and VEGFR-2 found in HemECs enhance affinity between the complexed proteins. In panel B, a schematic shows HemEC with lower VEGFR-1 levels, due to enhanced complex formation and decreased β_1 -integrin activity. This leads to increased VEGF-A availability and activation of VEGFR-2, resulting in endothelial proliferation.