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A switch in Notch Gene Expression Parallels Stem Cell to Endothelial Transition in Infantile Hemangioma

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Abstract

Background—Infantile hemangioma (IH) is the most common benign tumor of infancy, yet its pathogenesis is poorly understood. Notch family members are known to play a role in vascular development during embryogenesis and postnatal tumor angiogenesis, yet the role of Notch signaling in the pathogenesis of IH has not been investigated. This study aims to survey Notch expression in IH.

Materials & Methods—RNA from resected hemangioma tissue and hemangioma-derived stem cells (HemSCs) and endothelial cells (HemECs) were used for gene expression analyses by real-time PCR. Results were confirmed with immunofluorescence (IF) for protein expression in tissue.

Results—Real-time PCR showed that Notch family gene expression in IH is distinct from placenta and skin. Notch3 is expressed in HemSCs, but not in HemECs, indicating Notch3 is downregulated as HemSCs differentiate into HemECs. Moreover, expression of endothelial-associated Notch proteins, Notch1, -4, and Jagged-1 are increased in involuting hemangiomas and HemECs, suggesting that as hemangioma progresses towards involution, it acquires more differentiated endothelium. A subset of cells stained double positive for Notch3 and CD31, pointing to a potential intermediate between the HemSC cellular differentiation into HemEC.

Conclusion—HemSCs have distinct Notch expression patterns from differentiated HemECs and from normal human endothelial cells. Notch3 is expressed in HemSCs while Notch1, Notch4, and Jagged-1 have higher expression levels in HemECs. Notch3 was localized to the interstitial cells outside of the nascent vascular channels in proliferating IH tissue sections, but became more apparent in the perivascular cells in involuting IH. In summary, the pattern of Notch gene expression mirrors the progression from immature cells to endothelial-lined vascular channels (i.e., endothelial differentiation) that characterizes the growth and involution of IH.

Keywords

endothelial cell; infantile hemangioma; Notch gene expression; stem cell

Introduction

Infantile hemangioma (IH) is the most common benign tumor of infancy, but its pathogenesis and the mechanisms regulating its natural history are not well understood. IHs are known to go through rapid proliferation in the early neonatal period, followed by involution [1,2]. Many studies have characterized and analyzed IH tissues. IHs were first distinguished from other

vascular malformations by clinical history and histological characteristics [3]. The seminal paper by North et al. demonstrated that endothelial cells in IH stain positive for the glucose transporter-1 (GLUT1). GLUT1 is specifically expressed in IH as it is not found in other types of vascular tumors nor in vascular malformations; it is currently the gold standard for histological diagnosis of IH [4]. However, it is not known what the functional significance of GLUT-1 may be in the pathogenesis of IH. More recently, several studies have pointed to the molecular similarities between placenta and IH [5–7] that are not shared by other vascular tumors.

Other studies have documented differences in gene and protein expression between proliferating and involuting hemangiomas [8–12]. Proliferating and involuting hemangioma tissues have been analyzed by a variety of methods, including histopathological analyses, RT-PCR, and micro-array analyses, for differences in gene expression between the proliferating and involuting phases to identify potential regulators or mediators of hemangio-genesis. Some genes have been shown to have differential expression between proliferating and involuting hemangiomas: two examples are insulin-like growth factor 2 (IGF-2) [11] and indoleamine 2,3-dioxygenase (IDO) [10]. However, potential mechanisms on how these factors could regulate the phases of hemangioma have not been elucidated.

More recently, using laser capture micro-dissection and genome-wide transcriptional profiling, a list of genes expressed in the endothelial-lined vessels of hemangiomas in the proliferating and involuting phases was compiled and compared to gene expression in placental vessels. Cellular proliferation genes, such as Ki67, were found to be highly expressed in proliferating hemangiomas, whereas chronic inflammatory mediators, such as clusterin, were highly expressed in involuting hemangiomas [12]. Pertinent to this study, Jagged-1 and Notch4 were found to be increased 6.5- and 3.2-fold, respectively, in proliferating IH vessels compared to placental vessels [12].

There has been a steady debate as to whether intrinsic or extrinsic factors drive the pathogenesis of IH. The intrinsic theory of hemangioma origin postulates that the defect resides within cells of IH, in contrast to the extrinsic theory, which postulates that the surrounding environment contributes to the pathogenesis of IH [13]. Evidence that support the intrinsic theory includes studies that demonstrated that HemECs showed clonality, suggesting an intrinsic defect leading to formation of the lesion [14,15]. Moreover, HemECs were shown to have altered expression of the angiogenic regulator Tie2 [16]. An imbalance between VEGFR1 and VEGFR2 signaling in HemECs has also been uncovered which may contribute to the pathogenesis of hemangioma [17]. Evidence for endothelial progenitor cells (HemEPCs) in IH was first reported based on simultaneous expression of endothelial and progenitor cell markers [18]. Circulating endothelial progenitor cells were also reported in the blood samples from patients with IHs [19]. Both HemEPCs and HemECs showed properties of immature endothelial cell phenotype and behavior [20]. More recently, hemangioma-derived stem cells (HemSCs) have been isolated from proliferating IH and shown to differentiate into multiple lineages. Moreover, HemSCs implanted into nude mice differentiated into GLUT1-positive endothelial cells that had assembled into perfused blood vessels. To date, this is the first *in vivo* model that recapitulates, at least partially, the unique features of IH. Based on the *in vitro* multi-potentiality and *in vivo* model, the HemSC may be the cell of origin of IH [21].

Notch signaling has been shown to play a role in cell fate determination and arterial endothelial specification during embryogenesis [22–24]. In addition, Notch signaling also plays a role in post-natal tumor angiogenesis [25,26] and is an anti-angiogenic therapeutic target. Inhibition of delta-like ligand 4 (Dll4) signaling led to inhibition of tumor angiogenesis [27]. In addition, inhibition of the Notch signaling pathway has been shown to inhibit tumor angiogenesis in a mouse model [28]. Moreover, Notch proteins may exert their effects on vasculogenesis and

angiogenesis via VEGFR signaling [29,30]. Therefore, Notch may play a role in the pathogenesis of IHs via the VEGFR pathways. Preliminary studies by RT-PCR showed that Notch genes are expressed in hemangiomas [31]. This study will further investigate the expression of Notch receptors and ligands in hemangiomas.

Materials & Methods

Preparation of hemangioma specimen

IRB approval for collection of hemangiomas and control tissues were obtained from Columbia University College of Physicians & Surgeons (IRB #AAAA9976). Skin and placenta were chosen as control tissues. Skin was chosen as control, as resected IHs were cutaneous in origin; placenta was chosen based on the hypothesis of a placental origin of hemangiomas [6]. Tissues were either fixed in formalin or stored in *RNAlater* (Qiagen) and stored at -80°C until use.

RNA isolation and RT-PCR

Total RNA isolation was performed with RNeasy Mini Prep Kit (Qiagen). Quality of RNA was determined with spectrophotometry for optical density at 260/280nm. cDNA was synthesized by reverse transcription of 1–2 μg of total RNA, performed using the Superscript II system (Invitrogen). All reverse transcription (RT) reactions were probed for expression of the reporter gene β -actin to confirm successful generation of cDNA.

Real-time PCR

Real time PCR was performed with the Applied Biosystem 7300 and Syber Green Master Mix (Applied Biosystems) and done in triplicates. The PCR mixture consisted of Syber Green Master Mix, forward and reverse primers, and H_2O . The reaction mixture consisted of 57 μL of PCR mixture and 4.5 μL of cDNA, or 3 μL of real-time standards and 1.5 μL H_2O . Real-time standards were provided by Carrie Shawber (Columbia University). The PCR reaction was performed for 40 cycles with the following temperature cycles: 50°C (2 minutes) followed by 95°C (2 minutes) for initiation, followed by 40 cycles at 95 (15 seconds), 60 (40 seconds) and 72 (30 seconds). The results are normalized to β actin levels and the triplicate results averaged for each sample.

Primer sequence

Notch1 (sense) 5' CTC ACC TGG TGC AGA CCC AG 3', (antisense) 5' GCA CCT GTA GCT GGT GGC TG 3'; Notch2 (sense) 5' CAG TGT GCC ACA GGT TTC ACT G 3', (antisense) 5' GCA TAT ACA GCG GAA ACC ATT CAC 3'; Notch3 (sense) 5' CGC CTG AGA ATG ATC ACT GCT TC 3', (antisense) 5' TCA CCC TTG GCC ATG TTC TTC 3'; Notch4 (sense) 5' ATG ACC TGC TCA ACG GCT TC 3', (antisense) 5' GAA GAT CAA GGC AGC TGG CTC 3'; Jagged-1 (sense) 5' GCT TGG ATC TGT TGC TTG GTG AC 3', (antisense) 5' ACT TTC CAA GTC TCT GTT GTC CTG 3'; Dll4 (sense) 5' CGG GTC ATC TGC AGT GAC AAC 3', (antisense) 5' AGT TGA GAT CTT GGT CAC AAA ACA G 3', β -actin (sense) 5' CGA GGC CCA GAG CAA GAG AG 3', (antisense) 5' CTC GTA GAT GGG CAC AGT GTG 3'.

HemSC & HemEC

RNA from isolated HemSC and HemECs from 2 patients (Hem131 and Hem133) were provided by EB and JB and prepared according to previously published protocols [20,21]. cDNA synthesis and real-time PCR were performed with the same protocol as for hemangioma whole tissue. RNA from human dermal microvascular endothelial cells (HDMEC) was used as control. In addition, mesenchymal stem cells (MSC) were used as control RNA as well.

Immunofluorescence and double-staining

Immunofluorescence was performed for Notch3 staining, as well as for co-staining Notch receptors with CD31, a marker of endothelial cells. Briefly, the slides were de-paraffinized, and avidin and biotin were added with the blocking agents. The blocking agent was blotted off, and the first antibody was added to incubate overnight at 4°C. The slides were washed and incubated with the second antibody at room temperature for 30 minutes. Slides were incubated with immunofluorescent dye: Alexa fluor 488 (green) and 594 (red). Vectashield with DAPI (Vector) was applied to the slides and coverslip was placed, and the slides kept in dark. The antibodies used were: Notch3 (Abcam 23426, dilution 1:50) with 2nd antibody (goat anti-rabbit, dilution 1:500), Notch1 (Abcam 17843, pre-diluted; secondary antibody horse anti-mouse, dilution 1:200), Notch4 (Santa Cruz H225, dilution 1:100; secondary antibody goat anti-rabbit, dilution 1:500), Jagged-1 (R&D AF1277, dilution 1:200; secondary antibody rabbit anti-goat, dilution 1:300), CD31 (Dako, M0823, dilution 1:20; secondary antibody horse anti-mouse, dilution 1:300). All secondary antibodies from Vector Laboratories.

Statistical Analysis

For qPCR results, levels were measured in triplicate and normalized to β -actin for an assigned value and standard deviation. Statistical significance in the difference between 2 samples was calculated using Student's *t* test. A *p* value of 0.05 or less was considered statistically significant.

Results

Notch gene expression pattern in IHs are unique

We compared Notch expression pattern in IH, placenta and skin. Skin was chosen as most IHs are cutaneous—that is, in the epidermis and dermis. Previous studies have indicated molecular similarities between placenta and IH; lending support to the idea of a placental origin for IH [7]. Therefore placenta was chosen as a tissue for comparison to IH. All four Notch receptors Notch1–4, as well as 2 endothelial-associated ligands, Jagged-1, and Delta-like ligand 4 (Dll4), were studied.

Hemangioma—Both proliferating and involuting IH expressed Notch1, Notch3, Notch4, Jagged-1, and Dll4 mRNA. All transcript levels were higher than placenta. In addition, relative expression levels of Notch4 and Jagged-1 were higher in involuting hemangiomas when compared to proliferating hemangiomas. Notch1 and -3 expressions were comparable to skin, but the Notch4, Jagged-1, and Dll4 had higher levels when compared to skin. However, the transcript levels of Notch2 were lower in hemangiomas compared to both placenta and skin (Figure 1).

Placenta—The endothelial-specific Notch genes had low expression levels compared to IH. Notch2 expression levels were higher in placenta. In summary, the expression pattern in placenta was distinct from hemangiomas.

Skin—Skin had comparable levels of Notch1 and -3 expression to hemangiomas, but had relatively low levels of other endothelial-specific Notch genes (Notch4, Jagged-1 and Dll4). Similar to placenta, skin had high levels of Notch2 expression.

Notch expression in isolated HemSCs and HemECs in IH

Isolated HemSC and HemECs—In addition to whole tissue analyses, cells isolated from IHs—HemSCs and HemECs—were analyzed with the hypothesis that the changes seen between proliferating and involuting IH might be reflected in the isolated cell populations.

HemSC and HemECs isolated from hemangiomas in 2 patients (Hem131 and Hem133) were used.

HemSCs are multipotential stem cells that were shown to differentiate into endothelial cells and adipocytes. They were isolated initially by immunoselection using anti-CD133-coated magnetic beads and expanded in culture. HemSCs express the mesenchymal marker CD90 but not endothelial or hematopoietic markers. HemECs, on the other hand, are VE-cadherin and PECAM-1/CD31 positive endothelial cells from proliferating hemangioma that share *in vitro* characteristics with immature endothelial cells [20].

Notch3 is expressed in IH-derived stem cells (HemSC)

Isolated HemSCs had increased levels of expression of Notch3 when compared to the differentiated HemEC; (42- to 130-fold increase). Notch3 levels in HemSCs were also higher than human microvascular endothelial cells (HDMEC; 200–1300 fold), where levels were almost undetectable. Notch3 expression in HemSC was comparable to mesenchymal stem cells (MSC; 0.4–2.6 fold; data not shown) (Figure 2).

Notch2 was also highly expressed in HemSCs when compared to HemECs and HDMECs, parallel to Notch3 expression, further supporting the distinct phenotype of HemSCs.

In order to confirm real-time PCR results of Notch3 expression in IH tissue, immunofluorescence (IF) staining on proliferating and involuting IHS was performed. Notch3 positive cells were seen in hemangioma parenchyma, and in perivascular cells surrounding vessel lumens. By contrast, there was stronger and more distinct staining of Notch3 around the lumens in the involuting IHS (Figure 3). Co-staining for Notch3 and CD31 clearly showed that Notch3 was not present on endothelial cells but on cells surrounding the endothelium (arrows). However, there was a small subpopulation of cells that stained for both CD31+ and Notch3 (Figure 3, arrowheads).

Notch1, Notch4, and Jagged-1 expression is associated with involuting IHS and HemECs

HemECs had higher expression levels of Notch1, Notch4, Jagged-1, and Dll4 when compared to HemSCs, although gene expression levels of HemECs had some differences when compared to HDMEC. Namely, Notch1 levels were comparable, whereas Notch4 and Dll4 levels were lower than that found for HDMECs. Jagged-1 expression, on the other hand, was higher in HemECs than HDMECs. Importantly, the expression profile of Notch4 and Jagged-1 in HemSC and HemEC paralleled proliferating and involuting hemangiomas respectively (Figure 2).

These data suggest that involuting hemangiomas become “more endothelial” in nature, based upon the Notch genes expressed. Thus, we speculate that HemSC differentiation into HemECs coincides with involution and changes in Notch gene expression.

To confirm PCR findings, protein expression was probed by immunostaining. Notch1 and -4 receptors, and Jagged-1 were co-stained with CD31, a marker of endothelial cells. Immunofluorescence results show that the lumens of vessels are lined with CD31+ endothelial cells (luminal ECs) that also expressed these Notch proteins (Figures 4–6). Thus, they are expressed on endothelial cells. The lumens of proliferating specimens were less well-formed when compared to the involuting counterparts.

Discussion

Notch and Notch ligand expression in proliferating and involuting IHS are different and distinct from placenta and skin. Our data are consistent with Calicchio and colleagues, [12] who showed

that Notch4 and Jagged-1 expression were higher in proliferating IH when compared to placenta at the transcript level, although they did not analyze involuting IH. We here report a survey using both transcript and protein analysis on both proliferating and involuting IHs. In addition to the evaluation of Notch1, 4, and Jagged-1, we showed that Notch3 is primarily expressed by HemSC, with negligible levels in HemECs. This is consistent with previous studies, where Notch3 has not been reported to be expressed in endothelial cells, while its expression on mural cells has been well described [32,33]. The Notch3 expression seen here in HemSCs and in IH tissue sections suggest that, in IH, vascular smooth muscle cells, which are seen in postnatal arteries and arterioles and are positive for Notch3, may arise from HemSCs, and maintenance of Notch3 expression in HemSCs may allow their differentiation into smooth muscle cells or pericytes rather than endothelial cells.

Our finding of a sub-population of CD31/Notch3 doubly positive cells is consistent with other evidence that HemSCs give rise to HemECs, and we speculate that the doubly positive CD31/Notch3 cells represent a transient intermediate between HemSC and HemEC – perhaps the hemangioma endothelial progenitor cell, or HemEPCs, previously described [18]—that will fully differentiate into EC and eventually lose Notch3 expression. An alternative to this model proposes that loss of Notch3 expression may be a result of HemSC acquisition of an endothelial cell fate and thus Notch3 may not be causative of this transition.

By contrast, the endothelial-associated Notch1, -4, and Jagged-1 are detected at higher expression levels in HemECs and are localized in the lumen-forming endothelium. Khan and colleagues have shown that HemSCs can differentiate into HemECs [21]. Therefore, our data imply that differentiation of HemSCs into HemECs is associated with the expression of endothelial Notch proteins (Notch1/4). We speculate that Notch may play a role in such differentiation/progression or, in contrast, Notch expression may be activated in response to signals that drive the differentiation of HemEC. Future studies will focus on evaluation of Notch target gene expression, such as the Hes/Hey family of genes.

Even though HemECs had higher levels of expression of the endothelial-associated Notch ligands Jagged-1 and Delta-like ligand 4 (Dll4) when compared to HemSCs, those levels were different from HDMECs. The level of Jagged-1 expression was almost double that of HDMECs. On the other hand, even though Dll4 levels were higher in HemECs when compared to HemSCs, they are all lower than Dll4 in HDMECs. Benedito et al. recently demonstrated that Jagged-1 and Dll4 have opposing effects on angiogenesis. More specifically, Jagged-1 acts as a potent pro-angiogenesis regulator and inhibits Dll4-Notch signaling. Dll4 plays a role in selecting tip cells in sprouting and down-regulates VEGF signaling [34]. The high level of Jagged-1 expression in both HemSCs and HemECs in IHs is consistent with the pro-angiogenesis milieu of IHs. This imbalance of Jagged-1 and Dll4 levels may contribute to dysregulated vessel formation in this vascular tumor.

Proliferating hemangiomas are characterized as being hypercellular but one also finds areas with and without lumen formation. In contrast, involuting hemangiomas have less cellular density, but are characterized by prominent well-formed vascular channels or lumens [3]. This finding implies that becoming “more endothelial” coincides with the end of the growth phase and the beginning of the involuting phase, and the appearance of well-organized luminal structures. Notch3+ perivascular cells may need to interact with HemECs to form vascular channels. This could in part explain the well-formed lumens and vascular channels in involuting hemangiomas. Recent reports have shown that Notch3 expression in mural cells can be induced by co-culturing them with Jagged-1 expressing endothelial cell [35].

Future studies will concentrate on elucidating mechanisms that may regulate the maintenance or loss of Notch3 expression in HemSCs and hemangioma-associated pericytes, in governing

differentiation towards endothelial vs smooth muscle cell. In addition to fating cells towards a pericyte-smooth muscle cell fate, the role of Notch3 in maintaining pluripotency in HemSCs needs to be investigated.

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Figure 1. Notch expression profiles of proliferating and involuting infantile hemangiomas (IHs)
 When compared to placenta, both proliferating and involuting IHs had higher levels of gene transcripts for Notch1 ($p < 0.02$ and $p < 0.003$), Notch3 ($p < 0.025$ and $p < 0.0005$) and Notch4 ($p < 0.01$ and $p < 0.0005$), and involuting IHs showed higher expression levels of the Notch ligands Jagged-1 ($p < 0.0005$) and Delta-like ligand 4 (Dll4) ($p = 0.02$) when compared to placenta. Both proliferating and involuting IHs had higher Notch4 ($p < 0.002$ and $p = 0.0001$) and Dll4 ($p < 0.007$ and $p < 0.005$) mRNA expression when compared to skin. Furthermore, involuting IHs had higher Jagged-1 transcript levels ($p < 0.03$) compared to skin. By contrast, Notch2 expression was significantly higher in placenta ($p < 0.03$ and $p < 0.008$) and skin ($p < 0.005$ and $p < 0.005$) compared to both proliferating and involuting IHs. *statistical significance between IHs and placenta; # statistical significance between IHs and skin; \$ statistical significance between proliferating and involuting IHs.

Figure 2. Gene transcript levels Notch members in HemEC and HemSC

In isolated cells, Notch3 expression was 42–130 fold higher in HemSC when compared to HemECs or human dermal microvascular endothelial cells (HDMECs). On the other hand, HemECs expressed endothelial-specific Notch genes: Notch1, -4, Jagged-1, and Delta-like ligand 4 (Dll4) at higher levels than HemSC, in a similar pattern to involuting and proliferating hemangiomas, where higher levels of Notch4 and Jagged-1 are seen in involuting IH. When compared to HDMECs, HemECs had similar transcript levels of Notch1, approximately 50% of Notch4 transcripts, and twice the levels of Jagged-1 transcripts.

Figure 3. Notch3 expression does not co-localize with endothelial cells

(a–c) Proliferating hemangioma. CD31+ cells were located around the lumen (*red*), while Notch3+ cells were located around the lumen but not overlapping with endothelial cells (*green*; *arrows*). Interestingly, there was a subset of cells within the parenchyma that were positive for both CD31 and Notch3 (*yellow*, *arrowheads*). (d–f) Similarly, in involuting hemangiomas, endothelial cells (CD31+, *red*) were seen surrounding lumens, with Notch3+ cells (*green*) surrounding them without overlap. However, there were still Notch3+ cells (*green*) in the parenchyma (*arrows*), as well as Notch3/CD31+ cells (*yellow*, *arrowheads*).

Figure 4. Immunofluorescence co-staining of CD31 with Notch1
(a-c) Proliferating hemangioma. (d-f) Involuting hemangioma. Notch1 (green), CD31 (red) co-localize to peri-vascular structures (yellow). In addition, there were scattered endothelial cells within the parenchyma that also expressed Notch1.

Figure 5. Immunofluorescence co-staining of CD31 with Notch4
(a–c) Proliferating hemangioma. (d–f) Involuting hemangioma. Notch4 (green), CD31 (red). The Notch4 and CD31 positive cells co-localized to cells lining multiple small lumens (arrows), although some Notch4/CD31+ cells were within the parenchyma of the hemangioma. 11

Figure 6. Immunofluorescence co-staining of CD31 with Jagged-1
(a–c) Proliferating hemangioma. (d–f) Involuting hemangioma. Jagged-1 (*green*), CD31 (*red*). Similar to both Notch1 and –4, Jagged-1 co-localized with CD31+ endothelial cells surrounding lumens.