Genomic Imprinting of IGF2 Is Maintained in Infantile Hemangioma despite its High Level of Expression

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Hemangioma, the most common tumor of infancy, is characterized by rapid growth and slow regression. Increased mRNA expression of insulin-like growth factor 2 (IGF2) has been detected in the proliferating phase by cDNA microarray analysis, but the underlying mechanism causing the increase remains unknown. Here, using quantitative real-time polymerase chain reaction (PCR) and immunohistochemistry, we show that IGF2 is highly expressed in both proliferating and involuting phase hemangioma, but is not detectable in other vascular lesions such as pyogenic granuloma, venous malformation, lymphatic malformation, or in normal infant skin. Loss of imprinting of the Igf2 gene has been associated with IGF2 overexpression in a variety of childhood tumors. To determine if loss of imprinting and consequent bi-allelic expression might contribute to the increased expression of IGF2, we examined the genomic imprinting status of Igf2 in 48 individual hemangiomas. We determined allele-specific Igf2 expression using reverse transcriptase–PCR combined with analysis of an Apa I–sensitive restriction fragment length polymorphism. Similar to heterozygous normal skin controls, all 15 informative hemangiomas showed uniform mono-allelic expression of Igf2. Therefore, loss of imprinting is not involved in the increased expression of IGF2 in infantile hemangioma.

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INTRODUCTION

Infantile hemangioma proliferates rapidly during the 1st year of the child's life (proliferating phase), involutes slowly during the next 5 y (involuting phase), and regresses to fibrofatty tissue by 10 y of age (involuted phase) (1,2). Early proliferating hemangioma is characterized by active angiogenesis (3), presence of immature endothelial progenitors (4), and clonal expansion of endothelial cells with aberrant properties (5,6), yet the molecular cause of infantile hemangioma is unknown. Large hemangiomas can cause distortion or obstruction of vital structures and threaten life when located in the viscera. Therapy includes corticosteroid, interferon (7), vincristine (8), and, sometimes, surgical resection. However, these pharmacological treatments are not without side effects and complications (9–11). Targeted therapy will require a better understanding of the cellular and molecular basis underlying the pathogenesis of this tumor.

The human Igf2 gene, located on chromosome 11p15, encodes a mitogenic growth factor involved in embryonal and postnatal growth. In most normal human tissues, Igf2 is transcribed exclusively from the paternal allele resulting in mono-allelic expression (12). This rare form of genetic regulation is known as genomic imprinting. The precise mechanisms by which genomic imprinting occurs is an active area of investigation: epigenetic events, such as DNA methylation, have been described as well as the presence of *cis*-acting regulatory elements within the imprinted domain (13). It has been shown that hypermethylation of an imprinting-control region upstream of the H19 gene impedes access of the insulator protein CTCF (CCCTC-binding factor) to the maternal allele, thereby allowing specific activation of the paternal Igf2 allele (14,15).

Igf2 resides within a cluster of at least 12 imprinted genes on chromosome 11p15. Loss of imprinting (LOI) of Igf2 is a common epigenetic alteration in a variety of childhood neoplasms, such as Wilm's tumor, Ewing sarcoma, hepatoblastoma, rhabdomyosarcoma, congenital mesoblastic nephroma, and pediatric germ cell tumor (16,17). In addition, LOI of Igf2 has been shown to occur in 40% to 50% of patients with Beckwith-Wiedemann syndrome, a disorder characterized by somatic overgrowth and a predisposition to pediatric embryonal tumors (18). In some cases, LOI of Igf2 has been associated with overexpression of insulin-like growth factor 2 (IGF2) protein in childhood tumors (19–21).

Increased expression of IGF2 in proliferating hemangioma was 1st discovered by cDNA microarray analysis (22). In the present study, we found that IGF2 mRNA expression is induced in proliferating and involuting hemangioma tissues as compared with normal skin or other types of vascular anomalies, using both Northern blotting and quantitative reverse transcriptase–polymerase chain reaction (RT-PCR) techniques. In addition, we localized IGF2 protein to the vascular channels in proliferating and involuting hemangiomas, but found no IGF2 protein in the vasculature of normal infant skin, or in other vascular anomalies. Based on the reports of LOI of Igf2 in childhood tumors and overgrowth syndromes, we hypothesized that the high expression of IGF2 in infantile hemangioma might be caused by LOI of Igf2. Using *Apa*I

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polymorphism in exon 9 in the Igf2 gene (23), we showed that the genomic imprinting of Igf2 was maintained in the 15/15 informative hemangiomas. Our data indicate that the high expression of IGF2 in hemangioma is not associated with LOI of Igf2.

MATERIALS AND METHODS

Tumor Samples

Forty-eight resected cutaneous hemangioma specimens were obtained from patients 3 mo to 4 y of age under a protocol approved by the Committee on Clinical Investigation, Children's Hospital Boston (Boston, MA, USA). Clinical diagnosis of hemangiomas was confirmed by histological analyses performed in the Department of Pathology, Children's Hospital Boston. Additional vascular malformation and vascular tumor specimens (n = 9) were obtained from patients undergoing surgical resection at Children's Hospital Boston. Eleven neonatal foreskin controls were obtained in accordance with the Institutional Review Board at the Brigham and Women's Hospital (Boston, MA, USA). Immediately after resection, all the specimens were embedded in Optimal Cutting Temperature (OCT) compound and snap frozen in acetone/dry ice bath, or treated with RNA*later*™ solution (Ambion, Austin, TX, USA) and stored at –80 °C.

cDNA Microarray

Total RNA was isolated from proliferating hemangioma, involuting hemangioma, and normal human neonatal foreskin using RNeasy Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. Ten micrograms RNA from each tissue was reverse transcribed to cDNA, labeled with [α-^{33}P] dCTP, and hybridized separately to the GeneFilter GF211 (ResGen/Invitrogen Corp, Carlsbad, CA, USA). Gene expression patterns of 5184 genes were captured on a phosphor imager (Cyclone, Molecular Dynamics, Sunnyvale, CA) and analyzed by Pathways software (ResGen/Invitrogen Corp).

Northern Blotting and Immunohistochemistry

Detailed procedures for Northern blotting and immunohistochemistry have been described previously (24). Cryosections of tumor and normal tissues were either fixed in 4% paraformaldehye and labeled with mouse anti-human IGF2 (UBI, Lake Placid, NY, USA), or fixed in acetone and stained with rabbit anti-human GLUT1 (Dako, Carpinteria, CA, USA).

Quantitative RT-PCR

cDNA was synthesized from total RNA isolated from human full-term placenta, neonatal foreskin, proliferating hemangioma, lymphatic malformation, venous malformation, pyogenic granuloma, vascular tumors known as hemangioendothelioma, and human dermal microvascular endothelial cells with Superscript II RnaseH Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). DNase I digestion of RNA samples was performed prior to the reverse transcription. IGF2 was amplified by a TaqMan® real-time RT-PCR system using forward primer: 5-ACGTTCACTCT-GTCTCTCCCACTA-3 and reverse primer: 5-AATT CGTCT-GATTGTCCAGGGAGG-3 and Taqman probe: FAM 5-ACAGCT GACCTCATTTCCC GATACCT-3 TAMRA. Kinase insert domain receptor (KDR) was amplified by a TaqMan real-time RT-PCR system using forward primer: 5-TCAAAGGAGAAGCAGAGCCATGTG-3 and reverse primer: 5-GCACTCTTCCTCCAACTGCCAATA-3 and TaqMan probe: FAM 5-ACCACTCAAACGCTGACATG TACGGT-3 TAMRA. TaqMan probes and primers were designed using the PrimerQuest program from Integrated DNA Technologies (Coralville, IA, USA) and synthesized by Sigma Genosys Inc. Taqman real-time RT-PCR was performed for 55 cycles with Platinum Taq DNA Polymerase (Invitrogen) using the DNA Engine Opticon Monitor 2 System (MJ Research Inc, Waltham, MA, USA). Each PCR cycle was run at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, followed by extension at 72 °C for 10 min after 55 cycles. PCR products were sequenced (MRRC DNA Sequencing Core Facility, Children's Hospital Boston) to confirm the identity of the reaction products. A standard-curve quantitation method (25) was used to determine relative levels of RNA among the tissue specimens. Cycle threshold (Ct) values for KDR and IGF2 in serial dilutions of cDNA prepared from human dermal microvascular endothelial cells were plotted against the log of the serial dilution numbers. The resulting standard curves were used to calculate relative concentrations of IGF2 and KDR based on the Ct values in the different tissue specimens.

Hemangioma-derived Endothelial Cells (HemEC)

HemEC were isolated from proliferating hemangiomas and cultured as described (5) RNA from HemEC was isolated as described previously (24) and used for quantitative PCR as described above.

Genomic DNA Extraction

After specimens were minced, DNA was extracted using Wizard SV Genomic DNA Purification System (Promega, Madison, WI, USA) according to manufacturer's instructions.

Allele-specific Igf2 Expression Analysis

The genomic polymorphism of Igf2 in hemangiomas and normal skin controls was 1st evaluated using the *Apa*I restriction site in exon 9 of Igf2 (23). Next, RNAs from specimens found to be heterozygous were analyzed for allele-specific *Apa*-I site polymorphism by performing RT-PCR. To do this, 1 µg total RNA from each specimen was reverse transcribed with Superscript reverse transcriptase and oligo(dT) (Invitrogen). PCR amplification was performed on a PTC-100 Programmable Thermal controller (MJ Research Inc) using Taq DNA polymerase (Roche Applied Science, Indianapolis, IN, USA).

Oligonucleotide primers used were 5-CTTGGACTTTGAGT-CAAATTGG-3 (forward) and 5-GGTCGTGCCAATTACATTTCA-3 (reverse) (21). PCR conditions with genomic DNA as a template were 94 °C for 10 min, 35 cycles of 94 °C for 30 s, 55 °C for 1 min, and 72 °C for 30 s, followed by final extension at 72 °C for 10 min. When 0.5 mL of cDNA was used as a template, the annealing temperature was increased to 60 °C. Cycling numbers were optimized to ensure that products were not saturated. PCR was performed for 22 or 24 cycles. For each sample analyzed by RT-PCR, a negative control without reverse transcriptase was included to verify that there was no contamination with genomic DNA. PCR

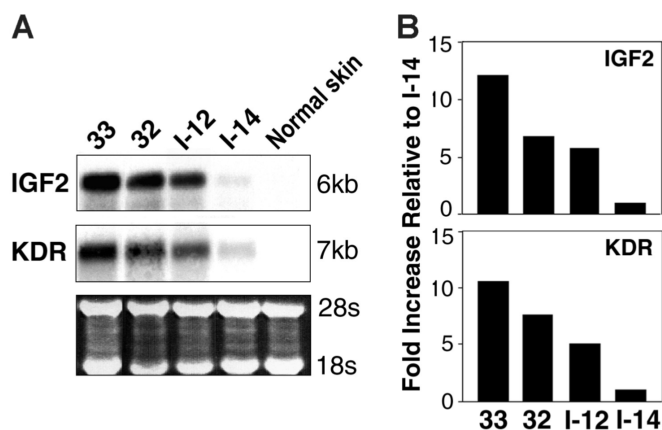

Figure 1. mRNA expression of IGF2 and KDR in infantile hemangioma. (A) Using ^{32}P-labeled cDNA probes to IGF2 and KDR, Northern blotting was performed on tissue RNA of proliferating hemangioma 33 and 32 from patients of 4 and 7 mo of age, involuting hemangioma I-12 and I-14 from 2 patients of 3 y of age, and normal infant skin. Equivalent loading of RNA was verified by staining the gel with ethidium bromide to visualize 28s and 18s rRNA. (B) The intensity of IGF2 and KDR signals were quantified, normalized to that of I-14, and plotted as relative fold increase.

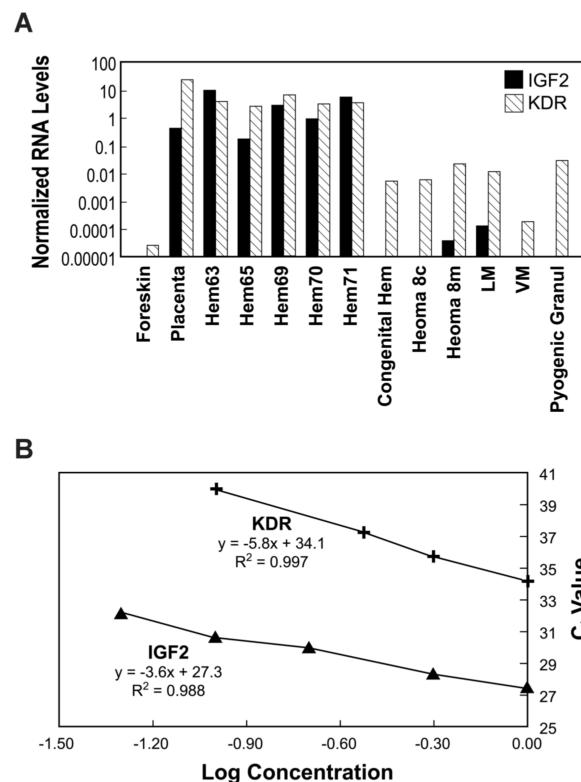

Figure 2. Quantitative real-time PCR analysis of IGF2 and KDR transcripts in hemangioma compared with levels in other vascular anomalies. In panel A, normalized RNA levels for IGF2 (black bars) and KDR (hatched bars) were determined from a standard curve generated from cycle threshold (Ct) values obtained from serial dilutions of cDNA from human dermal microvascular endothelial cells (panel B). Slopes were –5.8 for KDR (+) and –3.6 for IGF2 (–).

products were ethanol-precipitated, dissolved in double-distilled water, and digested overnight at 25 °C with 20 units of *Apa*I (New England Biolabs, Beverly, MA, USA). Digestion products were separated on a 2.5% ethidium bromide-stained agarose gel. The presence of both 292-bp and 227-bp fragments on the gel indicates heterozygosity at the Apa-I restriction site in the genomic DNA or bi-allelic RNA expression in the tissue.

RESULTS

Expression of IGF2 in Infantile Hemangioma

To screen for candidate genes with possible relevance to the pathogenesis of hemangioma, we performed cDNA microarray analysis of 5184 cDNA clones on a commercially prepared gene filter. Igf2 was identified among the up-regulated genes in proliferating and involuting phase tissues when compared with normal cutaneous controls. Expression patterns of Igf2 were further confirmed by Northern blotting of representative proliferating tumors designated 33 and 32 from patients 4 and 7 mo of age, respectively, as well as involuting specimens I-12 and I-14 from 2 patients, both 3 y of age (Figure 1A). The endothelial receptor for vascular endothelial growth factor, KDR, was analyzed as a relative measure of the endothelial content of the hemangioma. Normal infant skin was also analyzed as a negative control. Ribosomal RNA levels are shown to verify equivalent RNA loading and transfer. The decrease in IGF2 mRNA levels appeared to parallel the decrease in levels of KDR transcripts (see Figure 1A, 1B). The relative expression level of IGF2, therefore, seemed to be correlated with the endothelial content of the hemangioma specimen. I-12 was an early involuting hemangioma while I-14 was a late involuting specimen, resected from children of similar age, thus reflecting the variations frequently observed in the evolution of hemangioma. Neither IGF2 nor KDR were detected in normal infant skin (see Figure 1A). KDR was found by immunohistochemical staining in all tumor endothelial cells in the hemangioma specimens analyzed here (data not shown), which is consistent with our previous findings (4).

Quantitative PCR analysis of IGF2 in hemangioma compared with other vascular anomalies

To determine if Igf2 expression is unique to infantile hemangioma, we quantified IGF2 and KDR mRNA levels in a panel of proliferating hemangiomas, related vascular tumors and vascular malformation specimens for IGF2 and KDR mRNA using real-time quantitative PCR (see Figure 2A). A standard-curve quantitation method (25) was used to determine relative levels of the 2 mRNAs (see Figure 2B). Normalized RNA levels for IGF2 (black bars) and KDR (hatched bars) in normal human newborn foreskin (a negative control), normal human full-term placenta (a positive control), 5 different proliferating hemangiomas, and 6 specimens from other vascular anomalies are shown in Figure 2A. Congenital hemangioma is similar to infantile hemangioma (26). Hemangioendothelioma is a term applied to aggressive vascular tumors (27) that differ from the common infantile hemangioma because they do not undergo spontaneous involution. The lymphatic malformation specimen was from a 4-y-old child and the venous mal-

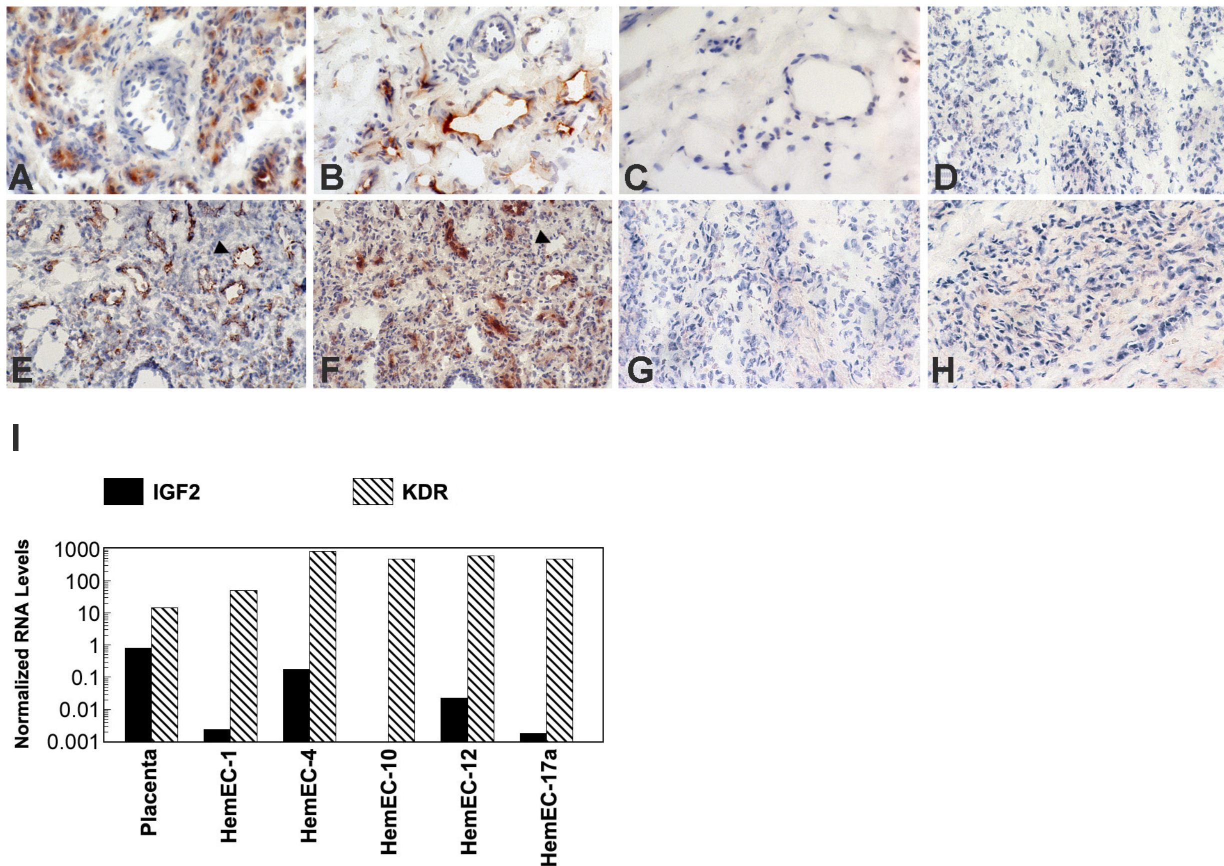

Figure 3. IGF2 protein expression in infantile hemangioma compared with other vascular anomalies. Cryosections of proliferating hemangioma (A and F), involuting hemangioma (B), normal infant skin (C), venous malformation (D), congenital hemangioma (G), and lymphatic malformation (H) were stained with a mouse anti-human IGF2 antibody (all panels except E). Note the presence of endothelial IGF2 expression associated with vascular channels and the lack of IGF2 associated with intralesional arterioles in hemangioma (A, B, and F). Panels E and F are serial sections of a proliferating hemangioma labeled with a rabbit anti-human GLUT1 antibody (E) for comparison to immunostaining observed with a mouse anti-human IGF2 antibody (F). Arrows in panels E and F highlight a GLUT1-positive vessel that appears to be IGF2-negative. (I) Normalized RNA levels for IGF2 (black bars) and KDR (hatched bars) in 5 different isolates of hemangioma-derived endothelial cells (hemEC) were determined by real-time PCR as described in Figure 2. Placental RNA served as a positive control.

formation was from an 18 y old. Pyogenic granuloma is vascular proliferation of the skin or mucosal membranes that is often called lobular capillary hemangioma. KDR was detected in all specimens tested. All 5 of the common infantile hemangioma specimens showed IGF2 levels similar to that detected in placenta, a tissue in which IGF2 is known to be expressed at high levels. In contrast, IGF2 was not detected, or detected at levels 4 orders of magnitude below levels in placenta and hemangioma, in tissue specimens from the other vascular anomalies, each of which can be distinguished from infantile hemangioma by the absence of GLUT1 immunostaining (28). Hence, the lack of IGF2 expression in these specimens, including congenital hemangioma (29), may prove to be an additional biochemical distinction between hemangioma and other vascular tumors and vascular malformations.

IGF2 protein is localized to vascular channels

Localization of IGF2 protein in hemangioma was determined by immunohistochemistry. IGF2 was detected on endothelial cells (EC) lining vascular channels, but not on EC of arterioles (Figure 3A, 3B) or arteries (not shown), in proliferating and involuting hemangioma. We failed to detect any IGF2 in involuted hemangioma specimens, which completely lacked residual tumor vessels (data not shown). Consistent with the results from Northern blotting and real-time PCR, IGF2 immunostaining was negative in normal infant skin (see Figure 3C). We also failed to detect any IGF2 in tissue sections from other types of childhood vascular lesions including congenital hemangiomas (Figure 3G) (n = 3), venous malformations (Figure 3D) (n = 2) and lymphatic malformations (Figure 3F), and pyogenic granuloma (n = 2) (data not shown).

For comparison with IGF2, a proliferating hemangioma was immunostained with antibody against human GLUT1 (see Figure 3E) (28). As previously reported, GLUT1 immunostaining was localized to the luminal surfaces of the vascular channels. In contrast, an adjacent section from the same hemangioma stained with anti-IGF2 (see Figure 3F) revealed a diffusely localized expression around the vessels and on some interstitial cells. We also observed a small subset of hemangioma endothelium that did not appear to have IGF2 immunoreactivity: compare arrows in Figure 3E (GLUT1-positive

Table 1. Allelic expression patterns of Igf2 in infantile hemangioma

Number[a]	Tissue	Age[b] (mo)/Sex	DNA[c]	RNA
1	Hem-32	7/F	B	
2	Hem-33	4/F	B	
3	Hem-35	5/F	A/B	B
4	Hem-36	11/M	B	
5	Hem-37	12/F	B	
6	Hem-38	7/F	A/B	B
7	Hem-39	7/M	A/B	B
8	Hem-40	24/F	B	
9	Hem-43	13/F	B	
10	Hem-45	24/F	A	
11	Hem-46	5/F	B	
12	Hem-47	12/M	B	
13	Hem-48	3/F	A	
14	Hem-50	15/F	B	
15	Hem-51	6/M	A/B	B
16	Hem-52	7/F	B	
17	Hem-53	10/F	A/B	B
18	Hem-54	12/F	B	
19	Hem-55	4/F	A/B	B
20	Hem-56	12/M	B	
21	Hem-57	4/M	A/B	B
22	Hem-58	3/M	B	
23	Hem-59	8/F	B	
24	Hem-61	2/F	B	
25	Hem-62	2/F	B	
26	Hem-63	5/F	A/B	B
27	Hem-64	12/M	B	
28	Hem (I-13)	17/M	B	
29	Hem (I-15)	24/F	B	
30	Hem (I-16)	60/F	A/B	A
31	Hem (I-17)	36/F	B	
32	Hem (I-18)	60/F	A/B	B
33	Hem (I-19)	36/F	A	
34	Hem (I-20)	29/F	A/B	A
35	Hem (I-21)	48/F	A/B	A
36	Hem (I-22)	36/F	B	
37	Hem (I-23)	24/F	A	
38	Hem (I-24)	24/F	A/B	A
39	Hem (I-25)	36/F	A/B	B
40	Hem (I-26)	36/M	A	
41	Hem (I-27)	36/F	A	
42	Hem (I-28)	48/F	B	
43	Hem (I-29)	12/F	B	
44	Hem (I-30)	24/F	A/B	B
45	Hem (I-31)	29/F	B	
46	Hem (I-32)	36/F	B	
47	Hem (I-33)	60/F	B	
48	Hem (I-34)	36/F	A	
49	F2	Newborn/M	B	
50	F3	Newborn/M	A/B	B
51	F4-9	Newborn/M	B	
52	F10	Newborn/M	A/B	A
53	F11	Newborn/M	A	

[a]1-27, proliferating hemangioma; 27-48, involuting hemangioma; 49-54, normal skin.
[b]Age is the age of the child at the time of resection.
[c]Alleles A and B of Igf2 denote *ApaI*-resistant and *ApaI*-sensitive alleles, respectively.

vessel) and in Figure 3F (IGF2-negative vessel in an adjacent section). This finding indicates that Glut1-positive hemangioma vessels have varying levels of IGF2 protein associated, perhaps due to varying degrees of vessel maturation seen in proliferating hemangiomas.

To determine if the IGF2 protein seen localized to the endothelium in hemangioma is expressed by HemEC, we measured IGF2 mRNA levels in 5 different HemEC isolates using the same real-time PCR methodology used in Figure 2. Relative levels of KDR were measured for comparison. In each of the 5 HemECs, IGF2 levels were 1 to 3 orders of magnitude lower than IGF2 in placenta (see Figure 3I). This result suggests that IGF2 expression has become down-regulated due to in vitro culture conditions, and, further, that the overexpressed IGF2 in hemangioma tissue results from extrinsic factors. An alternative possibility is that the endothelium is not the biosynthetic source of IGF2 in hemangioma: IGF2 protein, a secreted protein, may be synthesized by another cell type in the lesion and deposited on the endothelium, as seen in Figure 3. Further studies will be needed to determine which cells in hemangioma produce IGF2.

Genomic imprinting of Igf2 in infantile hemangioma

We examined hemangioma specimens for potential bi-allelic expression of Igf2 to determine whether loss of Igf2 imprinting was associated with elevated expression of IGF2. Genomic DNA from 48 hemangiomas and 11 normal cutaneous controls were analyzed for *Apa*I polymorphism in the exon 9 of Igf2 locus (23). Igf2 was amplified by PCR, digested with *Apa*I, and subjected to gel electrophoresis. Table 1 displays the results from all tissue specimens examined while Figure 4A shows representative PCR data from this analysis. Individuals with a heterozygous genotype showed 2 bands, 292 bp and 227 bp respectively, indicative of 2 alleles. Those with a homozygous phenotype were identified by the presence of one or the other single band: for example in specimens I-19 or I-13. Fifteen hemangiomas and 2 normal cutaneous specimens were heterozygous and thus informative for imprinting analysis of Igf2 (see Table 1).

cDNAs were prepared from all the informative tissues to analyze allele-specific expression of Igf2. All hemangioma specimens showed monoallelic expression of Igf2 as evidenced by the presence of 1 band after digestion of the cDNA-derived PCR product with Apa I. The 2 normal control cutaneous specimens (F3 and F10) also exhibited 1 band, indicating monoallelic expression. Homozygous hemangioma I-13 and skin specimens F4 and F11 were included to verify that *Apa*I digestions had gone to completion. The threshold for scoring LOI was less than 3:1 between the more-abundant and less-abundant alleles (30). Representative results are shown in Figure 4B. It is worth noting that in hemangioma specimens with heterozygous Apa I polymorphism, the frequency of the b allele (292 bp) was significantly higher than the a allele (227 bp), suggesting epigenetic heterogeneity.

DISCUSSION

We show here that IGF2 protein is strongly expressed in cells associated with the vascular channels of hemangiomas, but undetectable in normal skin or other types of human vascular anom-

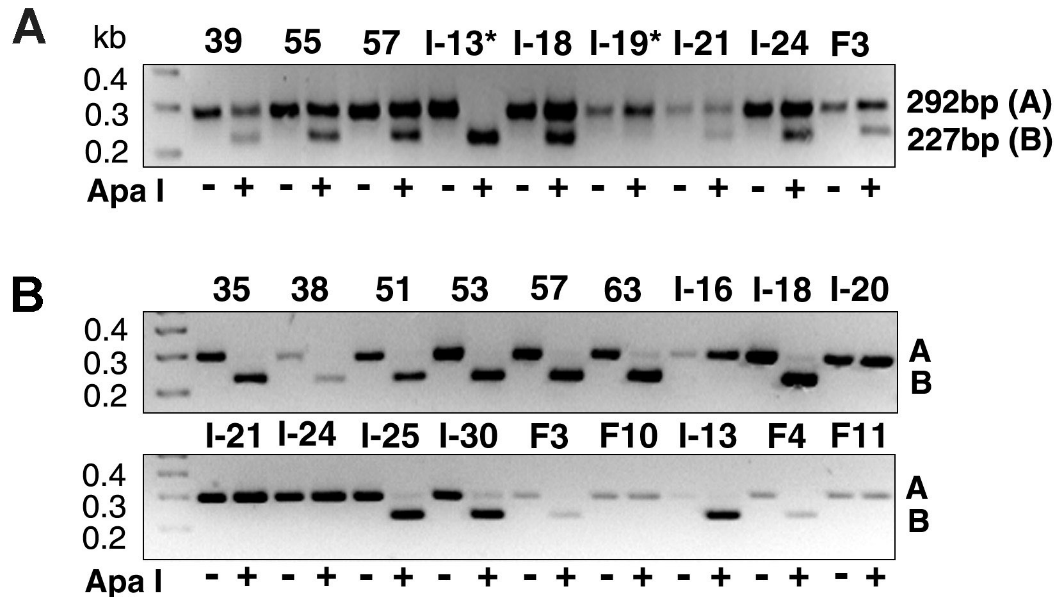

Figure 4. Imprinting status of Igf2 in infantile hemangioma. (A) Agarose gel electrophoresis of *Apa*I-digested PCR products of Igf2 exon 9 amplified from genomic DNA. Proliferating hemangioma designated 39, 55, 57, involuting hemangioma I-18, I-21, I-24, and normal infant skin F3 were heterozygous (A and B alleles). I-13 (B allele) and I-19 (A allele) were homozygous. The results are representative of 27 proliferating hemangiomas, 21 involuting hemangiomas, and 11 normal infant skin specimens (see Table 1). (B) Agarose gel electrophoresis of *Apa*I-digested cDNA products of Igf2. Monoallelic Igf2 expression is shown in proliferating hemangiomas 35, 38, 51, 53, 57, 63, involuting hemangiomas I-16, I-18, I-20, I-21, I-24, I-25, I-30, and normal infant skin control F3 and F10. The results are representative of the analysis of 15 informative cases. Homozygous hemangioma I-13 and skin control F4 and F11 were included to verify that *Apa*I digestions had gone to completion.

alies. The relative level of IGF2 mRNA in hemangioma correlates with the level of KDR, an endothelial-specific receptor for vascular endothelial growth factor (VEGF). Furthermore, the apparent induction of IGF2 in hemangioma is not caused by LOI and subsequent bi-allelic expression because mono-allelic expression was found in 15 of 15 informative hemangiomas.

The Igf2 gene is imprinted: i.e., the paternal allele is expressed while the maternal allele is silent. In addition to genomic imprinting, Igf2 can be regulated by transcription from multiple promoters, alternative translation initiation, and mRNA stability. Other components of the IGF system influence its biological activity, including 2 ligands (IGF1 and insulin), 6 high-affinity IGF-binding proteins (IGFBP-1 to IGFBP-6), and 2 IGF receptors (IGF-IR and IGF-IIR). The actions of IGFs may be modulated by the IGFBPs either in a positive or in negative way, depending on tissue type and physiological/pathological status (31,32). The detailed mechanisms of IGF2 regulation and function in infantile hemangioma remain unknown.

Despite our increased understanding of the cellular and molecular alterations in this most common childhood tumor, the primary event that initiates hemangioma-genesis remains to be identified. We and others showed that hemangioma-derived EC are clonal and exhibit abnormal behavior (5,6,24,33), suggesting the primary defect is due to a somatic mutation in a gene that regulates endothelial growth and differentiation. Increased TIE2 and decreased CD146 expression on hemangioma EC has been observed both in vitro and in vivo (24,33). In contrast, high IGF2 protein expression was found localized on or near EC in hemangioma tissues but IGF2 mRNA was detected only at very low levels in hemEC cultured in vitro (see Figure 3I). This suggests that IGF2 up-regulation is not an intrinsic property of clonal EC in hemangioma, but rather it is influenced by the extracellular matrix and cellular components in hemangioma.

Ritter and colleagues proposed a model in which IGF2 is an important regulator of the proliferating phase of hemangiogenesis, in part because they detected increased IGF2 in proliferating phase compared with involuting phase lesions (22). We found that the level of expression correlates with endothelial content in both proliferating and involuting phase, suggesting that the overall tissue level declines in the involuting phase because of the decreasing number of endothelial cells. An increase in endothelial apoptosis has been shown to coincide with the onset of the involuting phase (34). Therefore, we cannot exclude the possibility that IGF2 may play a role in the onset of apoptosis and thereby a role in the regression of hemangioma.

The precise role of IGF2 in the development of infantile hemangioma remains unknown. IGF2 may contribute to growth of hemangiomas by promoting angiogenesis in an autocrine or paracrine fashion. Two previous reports provide evidence that IGF2 directly induces angiogenesis by stimulating migration and morphological differentiation of human endothelial cells, as well as neovascularization in chick chorioallantoic membrane assay (35,36). Although IGF2 has been shown to regulate VEGF in other tumor settings, it is unlikely to affect VEGF function directly because many hemangiomas have been found to express little or no VEGF (22,24).

Our data show that the increased IGF2 expression is unique in infantile hemangioma. Therefore, IGF2 may prove to be an additional molecular marker for distinguishing hemangioma and other vascular anomalies. The expression pattern of IGF2 is similar to that of GLUT1 in hemangioma in that IGF2 is restricted to hemangioma vessels and is not detected on normal mature vessels such as arteries and arterioles around which hemangiomas form (28). Despite the presence of a small subset of $GLUT1^{+}$ $IGF2^{-}$ cells, IGF2 is mostly colocalized with GLUT1 in hemangioma. Thus, it is interesting to speculate that IGF2 may play a role in GLUT1 expression in hemangioma. These 2 proteins have been indirectly linked in previous studies as IGF2 was shown to induce expression of hypoxia-inducible factor 1a (37). Hypoxia-inducible factor 1a, in turn, is known to up-regulate GLUT1 (38). Further studies will be necessary to demonstrate a potential functional link between the elevated expression of IGF2 and GLUT1, and to determine the contributions of IGF2 to the pathogenesis of infantile hemangioma. In conclusion, our studies show that the imprinting status of Igf2 is maintained throughout the life-cycle of infantile hemangioma. These findings demonstrate that disruption of Igf2 imprinting does not play a role in hemangioma genesis.

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REFERENCES

1. Holmdahl K. (1955) Cutaneous hemangiomas in premature and mature infants. *Acta Paediatr*. 44:370-9.
2. Mulliken JB. (1993) Cutaneous vascular anomalies. *Semin. Vasc. Surg*. 6:204-18.
3. Takahashi K, Mulliken JB, Kozakewich HP, Rogers RA, Folkman J, Ezekowitz RA. (1994) Cellular markers that distinguish the phases of hemangioma during infancy and childhood. *J. Clin. Invest*. 93:2357-64.
4. Yu Y, Flint AF, Mulliken JB, Wu JK, Bischoff J. (2004) Endothelial progenitor cells in infantile hemangioma. *Blood* 103:1373-5.
5. Boye E, Yu Y, Paranya G, Mulliken JB, Olsen BR, Bischoff J. (2001) Clonality and altered behavior of endothelial cells from hemangiomas. *J. Clin. Invest*. 107:745-52.
6. Walter JW et al. (2002) Somatic mutation of vascular endothelial growth factor receptors in juvenile hemangioma. *Genes Chromosomes Cancer* 33:295-303.
7. Ezekowitz RA, Mulliken JB, Folkman J. (1992) Interferon alpha-2a therapy for life-threatening hemangiomas of infancy. *N. Engl. J. Med*. 326:1456-63.
8. Fawcett SL, Grant I, Hall PN, Kelsall AW, Nicholson JC. (2004) Vincristine as a treatment for a large haemangioma threatening vital functions. *Br. J. Plast. Surg*. 57:168-171.
9. Barlow CF et al. (1998) Spastic diplegia as a complication of interferon Alpha-2a treatment of hemangiomas of infancy. *J. Pediatr*. 132:527-30.
10. Worle H, Maass E, Kohler B, Treuner J. (1999) Interferon alpha-2a therapy in haemangiomas of infancy: spastic diplegia as a severe complication. *Eur. J. Pediatr*. 158:344.
11. Boon LM, MacDonald DM, Mulliken JB. (1999) Complications of systemic corticosteroid therapy for problematic hemangioma. *Plast. Reconstr. Surg*. 104: 1616-23.
12. Giannoukakis N, Deal C, Paquette J, Goodyer CG, Polychronakos C. (1993) Parental genomic imprinting of the human IGF2 gene. *Nat. Genet*. 4:98-101.
13. Arney KL. (2003) H19 and Igf2—enhancing the confusion? *Trends Genet*. 19: 17-23.
14. Rainier S et al. (1993) Relaxation of imprinted genes in human cancer. *Nature* 362:747-9.
15. Cui H et al. (2001) Loss of imprinting of insulin-like growth factor-II in Wilms' tumor commonly involves altered methylation but not mutations of CTCF or its binding site. *Cancer Res*. 61:4947-50.
16. Yun K. (1998) Genomic imprinting and carcinogenesis. *Histol. Histopathol*. 13: 425-35.
17. Schneider DT et al. (2001) Multipoint imprinting analysis indicates a common precursor cell for gonadal and nongonadal pediatric germ cell tumors. *Cancer Res*. 61:7268-76.
18. Weksberg R, Smith AC, Squire J, Sadowski P. (2003) Beckwith-Wiedemann syndrome demonstrates a role for epigenetic control of normal development. *Hum. Mol. Genet*. 12 Spec No 1:R61-8.
19. Roy RN, Gerulath AH, Cecutti A, Bhavnani BR. (2000) Loss of IGF-II imprinting in endometrial tumors: overexpression in carcinosarcoma. *Cancer Lett*. 153:67-73.
20. Muller S, Zirkel D, Westphal M, Zumkeller W. (2000) Genomic imprinting of IGF2 and H19 in human meningiomas. *Eur. J. Cancer* 36:651-5.
21. Ogawa O et al. (1993) Relaxation of insulin-like growth factor II gene imprinting implicated in Wilms' tumor. *Nature* 362:749-51.
22. Ritter MR, Dorrell MI, Edmonds J, Friedlander SF, Friedlander M. (2002) Insulin-like growth factor 2 and potential regulators of hemangioma growth and involution identified by large-scale expression analysis. *Proc. Natl. Acad. Sci. U.S.A*. 99: 7455-60.
23. Tadokoro K, Fujii H, Inoue T, Yamada M. (1991) Polymerase chain reaction (PCR) for detection of ApaI polymorphism at the insulin like growth factor II gene (IGF2). *Nucleic Acids Res*. 19:6967.
24. Yu Y, Varughese J, Brown LF, Mulliken JB, Bischoff J. (2001) Increased Tie2 expression, enhanced response to angiopoietin-1, and dysregulated angiopoietin-2 expression in hemangioma-derived endothelial cells. *Am. J. Pathol*. 159: 2271-80.
25. Ginzinger DG. (2002) Gene quantification using real-time quantitative PCR: an emerging technology hits the mainstream. *Exp. Hematol*. 30:503-12.
26. Boon LM, Enjolras O, Mulliken JB. (1996) Congenital hemangioma: evidence of accelerated involution. *J. Pediatr*. 128:329-35.
27. Enjolras O, Mulliken JB. (1998) Vascular tumors and vascular malformations, new issues. In: *Adv. Dermatol*. Paller AS (ed.) Mosby-Year Book, Inc., St. Louis, Mo, pp. 375-422.
28. North PE, Waner M, Mizeracki A, Mihm Jr. MC. (2000) GLUT1: a newly discovered immunohistochemical marker for juvenile hemangiomas. *Hum. Pathol*. 31:11-22.
29. North PE et al. (2001) Congenital nonprogressive hemangioma: a distinct clinicopathologic entity unlike infantile hemangioma. *Arch. Dermatol*. 137: 1607-20.
30. Cui H, Horon IL, Ohlsson R, Hamilton SR, Feinberg AP. (1998) Loss of imprinting in normal tissue of colorectal cancer patients with microsatellite instability. *Nat. Med*. 4:1276-80.
31. Jones JI, Clemmons DR. (1995) Insulin-like growth factors and their binding proteins: biological actions. *Endocr. Rev*. 16:3-34.
32. Le Roith D. (1997) Seminars in medicine of the Beth Israel Deaconess Medical Center. Insulin-like growth factors. *N. Engl. J. Med*. 336:633-40.
33. Li Q, Yu Y, Bischoff J, Mulliken JB, Olsen BR. (2003) Differential expression of CD146 in tissues and endothelial cells derived from infantile haemangioma and normal human skin. *J. Pathol*. 201:296-302.
34. Razon MJ, Kraling BM, Mulliken JB, Bischoff J. (1998) Increased apoptosis coincides with onset of involution in infantile hemangioma. *Microcirculation* 5: 189-95.
35. Kim KW et al. (1998) Insulin-like growth factor II induced by hypoxia may contribute to angiogenesis of human hepatocellular carcinoma. *Cancer Res*. 58:348-51.
36. Lee OH et al. (2000) Identification of angiogenic properties of insulin-like growth factor II in in vitro angiogenesis models. *Br. J. Cancer* 82:385-91.
37. Feldser D et al. (1999) Reciprocal positive regulation of hypoxia-inducible factor 1alpha and insulin-like growth factor 2. *Cancer Res*. 59:3915-8.
38. Chen C, Pore N, Behrooz A, Ismail-Beigi F, Maity A. (2001) Regulation of glut1 mRNA by hypoxia-inducible factor-1. Interaction between H-ras and hypoxia. *J. Biol. Chem*. 276:9519-25.