No Evidence for Maternal–Fetal Microchimerism in Infantile Hemangioma: A Molecular Genetic Investigation

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In this study, using the placental origin theory as a basis, we set out to explore whether hemangioma endothelial cells (HEC) were maternal in origin. We rigorously addressed this hypothesis using several molecular genetic techniques. Fluorescent *in situ* hybridization on surgical specimens of proliferating hemangiomas ($n=8$) demonstrated no XX-labeled HEC from resected tumors of male infants. This analysis was followed by PCR genotyping of HEC ($n=11$) using microsatellite markers where cellular components were genotyped and compared to genomic DNA of corresponding mother–child pairs. In the seven informative mother–child pairs, HEC matched the genotype of the child and not the maternal genotype. Concerned that HEC represented a mixed population of cells, we subsequently enriched for cells using the placental-specific endothelial cell (EC) marker, FcγRII. Three informative mother–child pairs exhibited only the genotype of the child in our enriched cell population. Using sequence analysis, we identified an informative single nucleotide polymorphism in an exon of the placental-EC-specific protein, GLUT1. When comparing GLUT1 complementary DNA (cDNA) with mother–child DNA, the genotype of the cDNA matched the constitutional DNA of the child. Our results indicate that hemangiomas are not microchimeric in origin. This study provides further insight into the origin of a tumor whose pathogenesis remains elusive.

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INTRODUCTION

Hemangiomas are the most common tumor of infancy, affecting a proportionately higher number of female infants than male infants. Although classified as a benign tumor, prevalence of the tumor, problems associated with its aberrant growth, and the lack of uniformly effective treatment options have warranted studies focusing on elucidating its origin.

Fundamental studies by North *et al.* (2000, 2001) identified the proteins, GLUT1, FcγRII, α2-laminin, and Lewis Y antigen, as uniquely expressed on the endothelial cell (EC) surface of infantile hemangiomas. These markers are also mainly expressed at high levels on blood vessels in the placenta. In studies performed subsequently, two other reports have shown ECs constituting hemangiomas are clonal in origin, suggesting clonal expansion from an endothelial precursor cell (Boye *et al.*, 2001; Walter *et al.*, 2002). These studies support the idea that the abnormal growth of hemangiomas is due to an intrinsic property of the endothelial component of the tumor. Collectively, these studies have offered support to a theory that hemangioma endothelial cells (HEC) acquire their placental phenotype owing to clonal expansion of embolized precursor ECs originating from placental vessels. Embolized placental ECs could reach fetal tissues via right-to-left shunts in the fetal circulation, where they would lodge *in situ* in the developing fetus. This placental origin hypothesis is further upheld by the observation that higher incidences of hemangiomas occur in children whose mothers have undergone chorionic villus sampling (Burton *et al.*, 1995). The placental origin hypothesis presents the possibility that the embolized ECs dislodged into the fetal circulation may be from either the fetal or maternal placental tissues.

The presence of a small number of maternal cells in fetal tissues has been termed maternal microchimerism. The clinical relevance of maternal microchimerism has been

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Abbreviations: cDNA, complementary DNA; EC, endothelial cell; FISH, fluorescent in situ *hybridization; HEC, hemangioma endothelial cell; mRNA, messenger RNA; PBL, peripheral blood leukocytes; SNP, single nucleotide polymorphism*

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shown in several disease states. Artlett *et al.* (2000) determined that maternal cells reside in the peripheral blood and muscle tissue of children suffering from juvenile idiopathic inflammatory myopathies. HLA genotyping revealed that maternal microchimeric cells persist in peripheral blood of immunocompetent individuals throughout their lives (Maloney *et al.*, 1999). In a pivotal study by Srivatsa *et al.* (2003) it was determined that maternal cells can migrate out of the fetal circulation into newborn tissues.

Because of the placental phenotype of HEC, maternal microchimerism may be hypothesized to be involved in the pathogenesis of hemangiomas. The purpose of this study was to investigate maternal microchimerism in hemangiogenesis. Proof that the tumor is maternally chimeric, would underline a potential mechanism for the aberrant growth of the tumor.

RESULTS

Our intial approach to exploring maternal microchimerism in hemangiomas was through fluorescent *in situ* hybridization (FISH) analysis. We performed X/Y FISH staining on serial sections of GLUT1+ proliferating hemangioma tissue from eight resected tumors of male infants. Normal foreskin was used as a control. No XX cells were identified within cells lining the capillaries of the hemangioma. The percentage of Y+ cells in HEC (Figure S1b, $67.3 \pm 5.3\%$) was not statistically different from the foreskin controls (Figure S1a, $70.5 \pm 7.1\%$, $P < 0.05$).

Our FISH results provided the foreground for a more robust analysis of maternal microchimerism. We employed microsatellite genotyping on fresh hemangioma tissue to identify the origin (parent or child) of the EC component of surgically resected tumors. Immediately after surgical resection, ECs from hemangiomas ($n = 11$) were magnetically sorted using EC-specific cell surface marker CD105 (endoglin sample 1–4) or CD31 (PECAM-1; samples 5–7). DNA was extracted from the ECs, as well as the overlying skin of the hemangioma, and peripheral blood leukocytes (PBL) of both patient and mother. PCR genotyping was performed using 12 microsatellite markers, six mapping to the X chromosome and six mapping to several autosomes. Seven of the 11 hemangiomas were informative for at least two markers (Table 1).

We sought evidence of maternal microchimerism, defined as a complete match between hemangioma-derived EC genotype and the mother's genotype. As represented in Figure 1a and 1b, for all seven informative mother–child pairs, the CD105 and CD31-selected ECs matched the constitutional genotype of the child also seen in hemangioma stromal cells and PBLs from the child. They did not match the

Table 1. Genotype analysis of CD105 or CD31-selected hemangioma cells compared to hemangioma stromal cells, genomic DNA of child, and genomic DNA of mother

				Genotypes			
Hemangioma	P or I	Informative markers	Chr. locus	Hemangioma EC	Hemangioma stromal cells	Genomic DNA of child	Genomic DNA of mother
1[1]	I	GATA42G01	X	AB	AB	AB	BC
		GATA027[2]	X	BB	BB	BB	AB
2[1]	P	ATCT057	X	BC	n.a.	BC	AB
		GATA42G01	X	BC	n.a.	BC	AB
		GATA027	X	BC	n.a.	BC	AB
3[1]	P	GATA10C11	X	BB	BB	BB	AB
		GATA42G01	X	BB	BB	BB	AB
4[1]	I	GATA42G01	X	BC	BC	BC	AB
		GATA32C12	7	AA	AA	AA	AB
		AGAT127	18	AB	AB	AB	BC
		GGAT2G03	3	BC	BC	BC	AB
5[3]	I	AGAT127	18	AB	AB	AB	AA
		ATAC037	7	AA	AA	AA	AB
6[3]	I	GGAA10C09N	14	AA	AA	AA	AB
	I	ATAC037	7	BC	BC	BC	AB
7[3]	I	GGAA10C09N	14	AB	AB	AB	AA
		ATAC037[4]	7	AC	AC	AC	BC

EC, endothelial cell.
P or I, proliferating or involuting hemangioma.
[1]CD105-selected EC.
[2]Data presented in Figure 1a.
[3]CD31-selected EC.
[4]Data presented in Figure 1b.

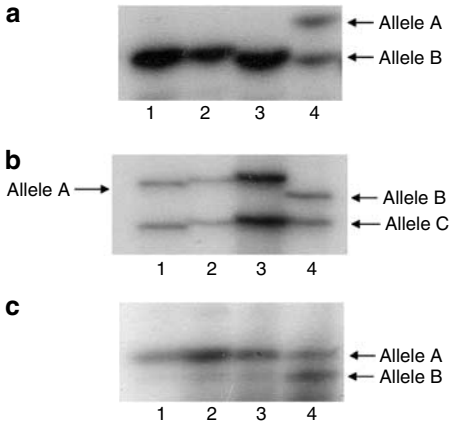

Figure 1. DNA from HECs do not exhibit the maternal genotype. (**a**, **b**). Genotype results for two representative hemangioma samples. ECs were magnetically sorted from hemangioma tissue using the EC-specific cell surface markers, CD105 (endoglin), or CD31 (pan-EC surface marker). Extracted DNA from sorted EC (lane 1) was genotyped using microsatellite markers for X chromosome or autosomes and compared with the genotype of DNA from overlying skin of the hemangioma (lane 2), and PBLs from the child (lane 3) and mother (lane 4). (**c**) ECs were isolated through FACS analysis using the hemangioma-specific EC marker CD32 and CD31. Extracted DNA from CD32/CD31-specific ECs (lane 1) was genotyped as before and compared with the genotype of DNA extracted from hemangioma CD31 cells (lane 2), and PBLs from both child (lane 3) and mother (lane 4).

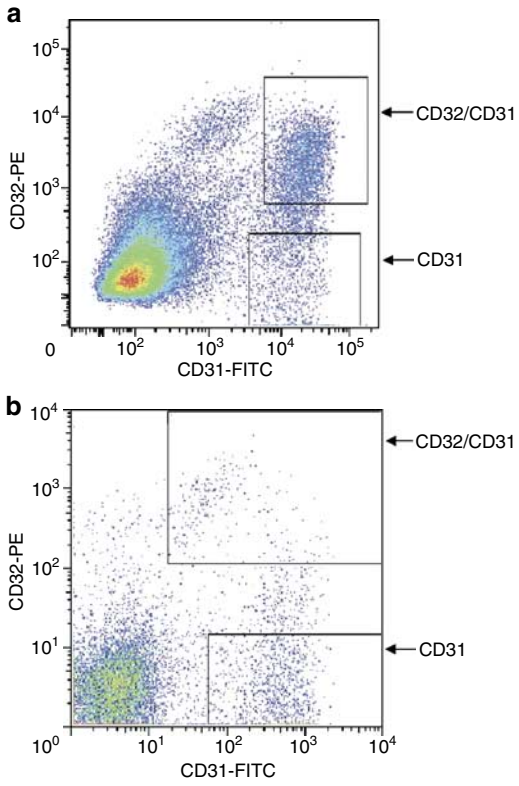

Figure 2. Proliferating hemangioma contains a greater number of CD32/CD31 double-positive cells compared to involuting hemangioma. Hemangioma cells were double-labeled with antiphycoerythrin CD32 and anti-FITC CD31. Cells were then sorted using flow cytometry. (**a**) A proliferating hemangioma (6% of gated cells are CD32/CD31-positive). (**b**) An involuting hemangioma (1% of gated cells are CD32/CD31-positive).

maternal genotype. As might have been expected, the maternal DNA always shared one allele with child's DNA (refer to both Table 1 and Figure 1). These preliminary results did not support maternal microchimerism.

Despite this result, we were concerned that the CD105 and CD31-selected ECs may have consisted of mixed population of ECs from the hemangioma proper and other ECs cell from surrounding vasculature. A mixed EC population could obscure the evidence of microchimerism by diluting the signal from the second maternal allele. We attempted to improve our analyses by enriching for ECs containing the placental expression phenotype. We attempted this enrichment in two distinct ways. The first study involved the use of FACS analysis to select for HEC that expressed the placental phenotype. Cells were sorted and selected for both CD31 and HEC marker FcγRII also known as CD32. The structure of CD32 with a large extracellular domain makes an ideal target for FACS analysis, enabling efficient and specific detection of the ECs derived from the tumor.

Total cell isolates from three hemangiomas (one proliferating and two involuting) were incubated with fluorescent-tagged antibodies to CD32 and CD31. As expected, a greater number of CD32/CD31-specific cells were sorted from a proliferating hemangioma (Figure 2a) compared to involuting (Figure 2b). PCR genotyping was performed using six microsatellite markers from the X chromosome. We compared the microsatellite repeat profile from DNA extracted from CD32/CD31-positive cells, CD31-positive cells, PBLs from the child, and PBLs from maternal blood (refer to Figure 1c). All three mother–child pairs were informative (Table 2). As previously observed, the hemangioma-derived ECs matched the constitutional genotype of the child, as found in the CD31 cells and PBLs from the child and not the maternal genotype.

In a second approach, we used the expression of the GLUT1 as a marker of the HEC. We reasoned that analysis of GLUT1 messenger RNA (mRNA) would effectively identify the origin of the HEC, provided that we could distinguish the mother and child's GLUT1 genotype. If maternal microchimerism was present then the GLUT1 mRNA expressed should resemble the maternal genotype at the mRNA complementary DNA (cDNA) level. cDNA was generated from unfractionated hemangioma tissue of six hemangiomas. Genomic DNA was extracted from PBLs from mother–child pairs. Each exon (nine total) from each sample was examined for a heterozygous single nucleotide polymorphism (SNP). Of six hemangiomas available for this analysis, only three were informative. A region of variance was found in exon 4 for all three pairs. The dbSNP rs11537641 (578C→T) was identified and results in a synonymous change, C133C. In each of the three cases, the informative allele expressed in the GLUT1 mRNA was similar to the patient's genomic DNA and not maternal genomic DNA (Figure 3).

Table 2. Genotype analysis of CD32/CD31-selected hemangioma cells compared to hemangioma CD31-positive cells, genomic DNA of child, and genomic DNA of mother

				Genotypes			
Hemangioma	P or I	Informative markers	Chr. locus	Hemangioma EC	Hemangioma stromal cells	Genomic DNA of child	Genomic DNA of mother
8	I	GATA10C11[1]	X	AA	AA	AA	AB
		AGAT104	X	AA	AA	AA	AB
		GATA42G01	X	BC	BC	BC	AB
9	P	GATA10C11	X	BC	BC	BC	AC
		AGAT104	X	CC	CC	CC	AC
10	I	GATA10C11	X	AB	AB	AB	BB
		GATA42G01	X	AB	AB	AB	AA

P or I; proliferating, or involuting hemangioma; EC, endothelial cells.
[1]Presented in Figure 1c.

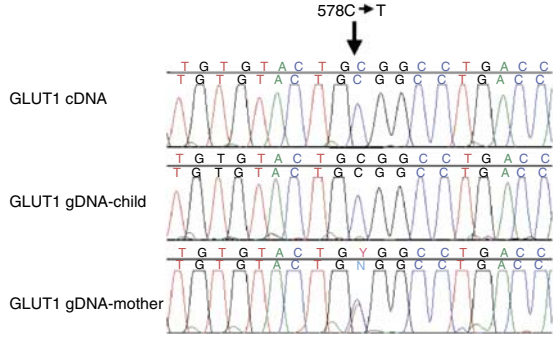

Figure 3. GLUT1 transcript from hemangioma tissue does not exhibit the maternal genotype. cDNA was generated from RNA extracted from hemangioma tissue, reverse transcribed, and PCR amplified. The amplified projects were subjected to DNA sequence analysis as a means to genotype the expressed GLUT1 transcript, which is only expressed with the hemangioma ECs. SNP rs1537461 in exon 4 is heterozygous in hemangiomas from this mother–child pair. GLUT1 cDNA and genomic DNA of the child are homozygous for the SNP-containing nucleotides CC at position 578. The mother, however, is heterozygous containing nucleotides CT in this same region.

DISCUSSION

The placental origin theory of hemangiogenesis maintains that ECs from placental vessel could be dislodged from the placenta, find residence in the developing fetus, and seed formation of a hemangioma that would proliferate rapidly after birth. This theory opens the possibility that the embolized ECs dislodged into the fetal circulation are either from fetal or maternal placental tissues. If HEC is derived from the maternal side, hemangiogenesis would be another medically-relevant example of maternal microchimerism. We set out to investigate this hypothesis through molecular genetic analysis of ECs derived from hemangiomas. We began with FISH where no XX cells were identified in serial sections of GLUT1$^+$ proliferating hemangioma tissue from eight resected tumors of male infants. Using PCR genotyping of multiple microsatellite markers, we identified 10 hemangiomas that were informative between the mother and her child, that developed a hemangioma. Our initial results using seven of these samples did not show evidence of the maternal genotype in the hemangioma-derived ECs. Concerned that our cell population was mixed, we attempted to enrich our HEC population. An additional three hemangiomas were subjected to more detailed analysis that amounted for genotyping only the ECs with the placental expression phenotype. In this instance, HEC were cell sorted both for an EC and a placental cell-surface marker. Using an alternative approach of expression genotyping, we identified a sequence polymorphism in the coding region of GLUT1, and then genotyped the transcript arising from the ECs. Both analyses confirmed the lack of a maternal genotype in the hemangioma-derived ECs.

The pathogenesis of hemangiomas has proven to be difficult to ascertain. The major key to its pathogenesis has been the strong correlation immunohistochemically between the placental vasculature and the ECs of the hemangioma. To date there has been at least seven markers identified that are coexpressed on placental and hemangioma vessels; GLUT1, merosin, Lewis Y antigen, Fcγ-RII, type III iodothyronine, indoleamine 2, 3 deoxygenase, and insulin-like growth factor 2 (Huang *et al.*, 2000; North *et al.*, 2000; North *et al.*, 2001; Ritter *et al.*, 2002; Huang *et al.*, 2003; Ritter *et al.*, 2003). Insulin-like growth factor 2 is expressed at high levels in hemangiomas, however, this high expression was found not be owing to a loss of imprinting (Yu *et al.*, 2004). Barnes *et al.* (2005) have recently strengthened the placental hypothesis with their study molecularly profiling hemangiomas and human placenta tissues. They identified three new genes, tissue factor pathway inhibitor 2, 17β-hydroxysteroid deyhdrogenase type 2 and γ *Drosophila* homolog-like 1. It was noted that 17β-hydroxysteroid dehydrogenase, γ *Drosophila* homolog-like 1 and insulin-like growth factor 2 are highly expressed in proliferative hemangiomas as opposed to involuting. They propose that the common genetic program shared between the placental vasculature and the EC of the hemangioma supports the placental origin theory and may correspond to a tumor that functions analogously to the placenta.

In conjunction with these findings, the results from our current study have provided greater insight into the placental phenotype of the tumor. We have determined that the phenotype is not owing to any maternal component that is represented in the tumor, and therefore the clonal expansion of ECs in the tumor stems from a cell type derived from the fetus. Other possible hypotheses, including mutation of ECs residing *in situ* or stimulation of an EC precursor cell in the fetus are also possible explanations to the phenotype.

MATERIALS AND METHODS

Samples

Blood samples and tissue specimens were obtained with informed consent from a total of 34 hemangioma surgeries conducted at either the University of North Carolina, Duke University, or New York University, Departments of Plastic and Reconstructive Surgery.

The Institutional Review Boards of the participating Universities approved the collection and use of these samples and experiments according to the Declaration of Helsinki Principles. Participants gave their written and informed consent. Hemangioma status, that is, proliferating or involuting was determined under physician guidance. Excluding archived tissue, 17 surgical specimens were obtained from female patients and nine were obtained from male patients (age range 4 months–3 years of age).

FISH

Hemangioma tissue from male infants and control foreskin were formalin-fixed at the time of excision, and subsequently paraffin-embedded ($n=8$). Surgical specimens were confirmed to be proliferating infantile hemangiomas by histopathology and GLUT1+ immunofluorescence. FISH analysis was performed on 5 μm serial sections of GLUT1+ proliferating hemangiomas and control specimens. Slides were deparaffinized with xylene for 10 minutes $\times$ 3, dried, and rinsed in 1 $\times$ sodium chloride sodium citrate buffer (Sigma, St Louis, MO). Sections were fixed in methanol/acetic acid (3:1) for 30 seconds, dried, and then treated with proteinase K, 10 μg/ml (Sigma), for 30 minutes at 37°C. Slides were incubated in 1% NP40 (Pierce, Rockford, IL) in phosphate-buffered saline for 30 minutes at 37°C. CEP X/Y DNA fluorescently labeled probes (Vysis, Des Plaines, IL) were applied to the sections as per manufacturer recommendations and then denatured at 80°C. Hybridization occurred overnight at 37°C in a humidified chamber, and we then removed non-hybridized probe with 50% formamide and 2 $\times$ sodium chloride sodium citrate buffer for 10 minutes at 65°C followed by 2 $\times$ sodium chloride sodium citrate buffer and 2 $\times$ sodium chloride sodium citrate buffer/0.5%, NP40 for 5 minutes at 45°C. Slides were counterstained with 4, 6-diamidino-2-phenylindole, and mounted with Vectashield (Vector Laboratories, Burlingame, CA). FISH signals for X/Y chromosomes were enumerated using an Olympus microscope equipped with a triple-pass filter.

Magnetic cell sorting

Immediately after surgery, hemangioma tissue was finely minced under sterile conditions and placed in a 10 ml conical with a 1:5 dilution of Liberase Blendzyme 3 (Roche-Applied Science, Indianapolis, IN) in 1X phosphate-buffered saline. Conical was placed in rocker at 37°C for 10–20 minutes or until a slurry was formed. Cell slurry was filtered using at first a 70 μm cell strainer and followed with a 40 μm cell strainer (BD Biosciences, San Jose, CA). Filtered slurry was then centrifuged for 10 minutes at 200 $\times$ g. Cells were rinsed and prepared for CD105 or CD31 sorting magnetically according to company instructions (Miltenyi Biotec, Gladbach, Germany).

PCR genotyping

Polymorphic tetranucleotide markers were chosen from the X chromosome and autosomes based upon heterozygosity score greater than 0.60 (Marshfield Clinic Research Foundation, Marshfield, WI). Genomic DNA was extracted from CD105 or CD31 magnetically sorted cells and PBL of mother and child using Puregene DNA extraction kit (Gentra Systems, Minneapolis, MN). Either the forward or reverse primer was incubated in a kinase reaction containing T4 polynucleotide kinase, kinase buffer (Invitrogen Life Technologies, Carlsbad, CA) in an excess of γP32 ATP (ICN Biomedicals, Costa Mesa, CA). Radiolabeled primer was then added to PCR reaction mix containing 50 ng of DNA, PCR reaction mix, Platinum Taq Polymerase (Invitrogen Life Technologies). Routine PCR was performed with conditions varying with each tetranucleotide marker. PCR products were run on a 4% non-denaturing polyacrylamide gel. The gel was dried and exposed to Kodak X-Omat LS film (Eastman Kodak, New Haven, CT).

FACS sorting

Cell sorting of HECs using CD32 and CD31 cell surface markers was achieved using flow cytometry. A single cell suspension was generated as described above for magnetic cell isolation. Cells were incubated with both FITC-labeled mouse anti-human CD31 and phycoerythrin-labeled mouse anti-human CD32 (BD Pharmingen, San Diego, CA). Cells were stained on ice in the dark for 30 minutes. Background fluorescent was controlled for using anti-mouse CompBeads (BD Pharmingen).

GLUT1 SNP analysis

GLUT1 gene sequence was obtained through the UCSC genome browser (http://genome.ucsc.edu, GenBank accession number NM_006516). Heterozygous SNP detection for genomic DNA was performed by designing primers for each of the nine exons around their corresponding flanking intronic sequence. Each exon was amplified by PCR using 100 ng of genomic DNA extracted from PBLs of mother and child described above. For mRNA expression analysis, primers for coding exons were designed across intronic sequences to ensure purity of total RNA extracted (primers available upon request). For mRNA expression analysis, unfractionated hemangioma tissue was placed in Trizol (Invitrogen Gibco BRL, Carlsbad, CA) immediately after surgery and homogenized using a Polytron homogenizer. Total RNA was extracted and cDNA was amplified using 1–5 μg of total RNA combined in a tube with random hexamers, oligo dTs, Monkey Moloney Leukemia Virus reverse transcriptase and 5X First Strand buffer (Invitrogen Life Technologies). PCR products for both genomic DNA and cDNA were loaded and run on 1% Agarose (Amresco, St Louis, MO) using 0.5% Tris-borate EDTA. Products were eluted from the gel by freezing and then centrifuging at 18,000 $\times$ g for 10 minutes. Exons were sequenced using the ABI PRISM Big Dye terminator chemistry (PE Biosystems, Foster City, CA).

CONFLICT OF INTEREST

The authors state no conflict of interest.

SUPPLEMENTARY MATERIAL

Figure S1. FISH analysis on tissue sections from hemangiomas resected from male infants.

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