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Mesenchymal Stem Cells and Adipogenesis in Hemangioma Involution

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ABSTRACT

Hemangioma is a benign tumor of infancy whose hallmark is rapid growth during the first year of life followed by slow regression during early childhood. The proliferating phase is characterized by abundant immature endothelial cells, the involuting phase by prominent endothelial-lined vascular channels and endothelial apoptosis, and the involuted phase by few remaining capillary-like vessels surrounded by loose fibrofatty tissue. Nothing is known about the mechanisms that contribute to the adipogenesis during this spontaneous regression. We postulated that mesenchymal stem cells (MSCs) reside in the tumor and preferentially differentiate into adipocytes. To test this hypothesis, we isolated MSCs from 14 proliferating and five involuting hemangiomas by taking advantage of the well known selective adhesion of MSCs to bacteriologic dishes. These hemangioma-derived MSCs (Hem-MSCs) are similar to MSCs obtained from human bone marrow, expressing the cell surface markers SH2 (CD105), SH3, SH4, CD90, CD29, smooth muscle α-actin, and CD133 but not the hematopoietic markers CD45 and CD14 or the hematopoietic/endothelial markers CD34, CD31, and kinase insert domain receptor (KDR). Hem-MSCs exhibited multilineage differentiation with robust adipogenic potential that correlated with the proliferating phase. The numbers of adipogenic Hem-MSCs were higher in proliferating-phase than in involuting-phase tumors and higher than in normal infantile skin. Furthermore, Hem-MSCs exhibited a random pattern of X-chromosomal inactivation, indicating that these cells are not clonally derived. In summary, we have identified MSCs as a novel cellular constituent in infantile hemangioma. These MSCs may contribute to the adipogenesis during hemangioma involution. STEM CELLS *2006;24:1605–1612*

INTRODUCTION

Infantile hemangioma is the most common tumor of infancy, affecting about 10% of white babies [1–3]. The lesions appear early in post-natal life, grow rapidly during infancy, and involute spontaneously in early childhood. The life span of infantile hemangioma is divided into proliferating phase (birth to 1 year of age), involuting phase (1–5 years), and involuted phase (5–10 years) [2, 4]. Although the precise cause of infantile hemangioma remains unknown, progress has been made toward understanding the cellular and molecular events that occur during its life cycle. The early proliferating tumor is characterized by angiogenesis [4–6], the presence of endothelial progenitors [7], and clonal expansion of endothelial cells [8, 9] with abnormal properties [8, 10, 11]. Genetic alterations in rare kindreds have been reported [12, 13]. Somatic mutations of vascular endothelial growth factor receptors have also been reported in some hemangiomas [9].

Less is known about the mechanisms that initiate the involution of hemangioma. An increase in apoptosis of endothelial cells [14, 15] and downregulation of anti-apoptotic genes [15, 16] coincide with the onset of regression. The appearance of adipocytes in the involuting phase and the accumulation of fibrofatty tissue in the involuted phase is seen in histological sections. In some cases, the aggregate of fatty residium seems to

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clinically correlate with the size of the tumor. Current pharmacologic therapies target the proliferating endothelium, not adipogenesis. It is intriguing to speculate that identifying regulators of cell differentiation in hemangioma may provide mechanisms for accelerating involution and, perhaps, for manipulating cellular differentiation to minimize tissue destruction and residual disfigurement.

We hypothesized that mesenchymal stem cells (MSCs) would be likely precursors of the adipocytes in hemangioma. MSCs are defined by their self-renewal capability and potential for multilineage differentiation into adipocytes, osteoblasts, chondrocytes, myocytes, neuronal cells, and hepatocytes [17]. Human MSCs were originally isolated from bone marrow (BM) [18]. Peripheral blood and umbilical cord blood have since emerged as additional sources [19–21]. Studies have shown that in adults, MSCs reside in many connective tissue sites, including (but not limited to) dermis, muscle, adipose, trabecular bone, and synovial membranes [17]. After transplantation of human MSCs in fetal sheep and blastocytes of mice and nonhuman primates, the cells have been found to engraft and undergo site-specific differentiation in tissues such as lung, bone, spleen, skin, and brain in normal or disease settings [22–26]. Based on this diverse pattern of localization, it is reasonable to postulate that MSCs or mesenchymal progenitors are present in hemangioma and differentiate into adipocytes following specific cues from the microenvironment during the involuting and involuted phases.

In this study, we isolated MSCs from 14 proliferating and five involuting hemangiomas by taking advantage of the well characterized selective adherence of MSCs to plastic culture dishes. We showed that hemangioma-derived MSCs (Hem-MSCs) were morphologically and immunophenotypically similar to MSCs from human BM. In appropriate induction media, Hem-MSCs were induced to differentiate into adipocytes, osteoblasts, or myoblasts. The number of adipogenic cells was strikingly higher in Hem-MSCs from proliferating hemangiomas when compared with involuting hemangiomas or with normal infant skin. Unlike clonal hemangioma-derived endothelial cells (Hem-ECs) we described previously [8], Hem-MSCs were not clonal when assayed for X-chromosome inactivation. The identification of MSCs in hemangioma and their adipogenic propensity provide new information on the cellular basis of hemangioma.

Materials and Methods

Tissue Specimens

Cutaneous hemangiomas and pyogenic granulomas (PGs) were obtained with approval from the Committee on Clinical Investigation, Children's Hospital Boston (Boston, http://www.childrenshospital.org). Clinical diagnosis of hemangioma was confirmed in the Department of Pathology, Children's Hospital Boston. Normal neonatal foreskins were obtained in accordance with the Institutional Review Board at the Brigham and Women's Hospital (Boston, http://www.brighamandwomens.org). See supplemental online Table 1 for additional information on tissue specimens.

Isolation and Culture of MSCs

Tissue specimens, taken immediately after resection, were rinsed three times in phosphate-buffered saline (PBS), minced, and digested with 1 mg/ml collagenase A (Roche, Indianapolis, http://www.roche.com) in PBS containing 0.1% bovine serum albumin (BSA) at 37°C for 1 hour. Tissue was homogenized and filtered through 100- and 40-μm cell strainers (Fisher Scientific, Pittsburgh, http://www.fishersci.com) to obtain a single-cell suspension. Red blood cells were lysed in ammonium chloride (StemCell Technologies, Vancouver, BC, Canada, http://www.stemcell.com). Cells were plated on noncoated "bacteriologic" Petri dishes at $1.5 \times 10^5/cm^2$. After 12 hours, floating cells were removed by washing three times with PBS. The attached cells were cultured at 37°C in 5% CO_2 in Dulbecco's modified Eagle's medium/high glucose (GlutaMAX™ Media) supplemented with 20% embryonic stem cell-qualified fetal bovine serum, 0.55 mM β-mercaptoethanol, 1% glutamine-penicillin-streptomycin (Invitrogen, Carlsbad, CA, http://www.invitrogen.com), and minimal essential media (MEM) nonessential amino acid solution (100×) (Sigma, St Louis, http://www.sigmaaldrich.com). The cells could be expanded up to 15 passages.

Flow Cytometry

Cells were harvested with 0.05% trypsin/0.53 mM EDTA (Invitrogen) and resuspended in PBS containing 2 mM EDTA and 0.5% BSA. Single-cell suspensions were labeled with fluorescein isothiocyanate (FITC)-conjugated antibodies against human CD45, CD14 (BD PharMingen, San Jose, CA, http://www.bdbiosciences.com/pharmingen), CD34 (Miltenyi Biotech, Auburn, CA, http://www.miltenyibiotech.com), CD29 (Beckman Coulter, Miami, http://www.beckmancoulter.com), and/or PE-conjugated antibodies against human CD90, CD105 (BD PharMingen), CD133 (Miltenyi Biotech), and CD31 (Chemicon International, Temecula, CA, http://www.chemicon.com). FITC-conjugated anti-human KDR was prepared as previously reported [7]. To establish background staining, cells were labeled with FITC- or PE-conjugated isotype-matched control antibodies. Samples were analyzed on a FACSVantage SE flow cytometer (BD PharMingen). Ten thousand to 50,000 events were acquired in list mode with CellQuest software (BD PharMingen). The list mode files were analyzed with WinMDI software (The Scripps Research Institute, La Jolla, CA, http://www.scripps.edu).

Immunofluorescent Staining

Indirect immunofluorescence was performed as described previously [8]. Briefly, cells were plated into eight-well chamber slides (Nalge Nunc International, Naperville, IL, http://www.nalgenunc.com) for 24 hours prior to staining, washed with PBS, fixed in −20°C methanol for 6 minutes, and washed three times with PBS. Slides were incubated for 1 hour at room temperature with mouse anti-human CD4, CD8, CD14, CD34, CD45, CD105, and CD90 antibodies (BD PharMingen), mouse anti-human CD29 (Beckman Coulter), anti-α-smooth muscle actin monoclonal antibody (Sigma), anti-human calponin (Dako Corp., Carpinteria, CA, http://www.dako.com), goat anti-human CD31, CD117 (c-kit) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, http://www.scbt.com), and the monoclonal antibodies SH3 and SH4 (kindly provided by Dr. William T. Tse, Children's Hospital Boston). As

controls, cells were incubated with isotype-matched IgGs. After labeling with FITC-conjugated anti-mouse or anti-goat IgG (Vector Laboratories, Burlingame, CA, http://www.vectorlabs.com), slides were mounted with DAPI (4′,6-diamidino-2-phenylindole) mounting medium (Vector Laboratories) and examined by fluorescence microscopy using a Nikon microscope and IP Lab imaging software (Nikon, Tokyo, http://www.nikon.com).

Differentiation of MSCs

MSCs were plated at the same density in 35-mm tissue culture dishes for each lineage differentiation assay. Twelve hours later, cells were differentiated into adiopocytes, osteocytes, or myocytes in specific induction media as described previously [27]. Medium was changed every 3 days. After induction for 0, 8, or 16 days, cells were stained with Oil Red O to detect adipocytes or with silver nitrate (von Kossa method [27]) to detect calcium deposition indicative of osteogenesis or were immunostained with anti-skeletal muscle myosin heavy chain (Sigma) to detect myocytes.

Proliferation of MSCs

Hem-MSCs were plated at a density of 1×10^4 cells per well into 24-well plates in MSC culture medium. Cell numbers were counted at days 0, 3, 6, and 10 with a Coulter counter (Beckman Coulter). Cell viability was assessed by the ability of the cells to exclude trypan blue (Invitrogen).

Relative Quantitative Reverse Transcription-Polymerase Chain Reaction

Total RNA was isolated from Hem-MSCs and control MSCs were isolated from newborn foreskin using RNeasy Mini Kit (Qiagen, Valencia, CA, http://www1.qiagen.com). cDNA was synthesized with Superscript II RNaseH Reverse Transcriptase (Invitrogen). DNase I digestion of RNA was performed prior to the reverse transcriptase reaction. Peroxisome proliferator-activated receptor-γ-2 (PPAR-γ-2) was amplified by polymerase chain reaction (PCR) using a forward primer (5′-GCTGTTATGGGTGAAACTCTG-3′) and a reverse primer (5′-TCGCAGGCTCTT TAGAAACTC-3′). 18s rRNA was amplified as an internal control using primer pairs from QuantumRNA Classic II 18S Internal Standard (Ambion, Austin, TX, http://www.ambion.com). Hot-start PCR was performed, and each PCR cycle was run at 94°C for 45 seconds, 58°C for 45 seconds, and 72°C for 90 seconds followed by an extension at 72°C for 10 minutes.

Clonality Assay

Genomic DNA was extracted from Hem-MSCs and analyzed by the human androgen receptor (HUMARA) assay as described previously [8].

RESULTS

Isolation and Cell Surface Antigenic Profile of MSCs from Hemangioma

Infantile hemangioma starts as a rapidly growing tumor composed of densely packed endothelial and stromal cells. During the course of involution, the tumor slowly transforms into fibrofatty tissue with abundant adipocytes in situ (Fig. 1). To examine our hypothesis that MSCs are present and give rise to adipocytes in hemangioma, we isolated MSCs from homogenates of hemangioma tissue by taking advantage of the well characterized selective adherence of MSCs to plastic [17]. Adherent cells were readily obtained from 14 proliferating tumors from patients 3 to 18 months of age and from five involuting tumors from children 1 to 4 years of age (supplemental online Table 1). PG, also called lobular capillary hemangioma, is another common vascular lesion of the skin that differs from infantile hemangioma in its clinical and histological features [28, 29]. In contrast to hemangioma, attached cells were not found in two different PGs.

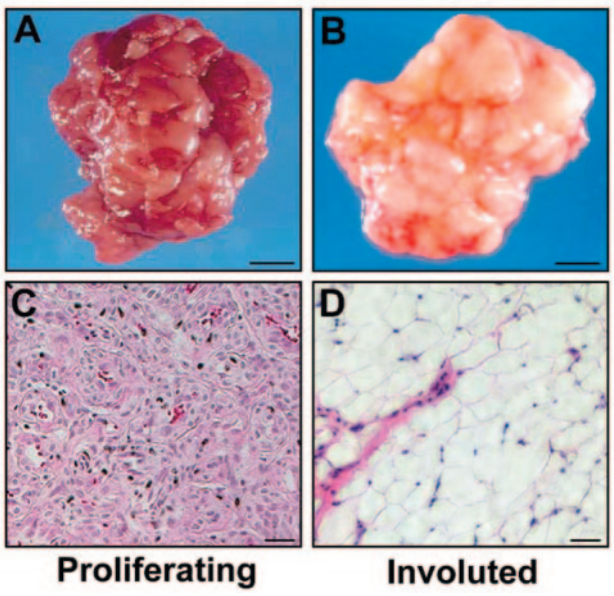


Figure 1. Adipocytes in involuted hemangioma. **(A):** Photograph of a freshly resected proliferating hemangioma from a 3-month-old child. **(B):** Photograph of an involuted hemangioma from a 7-year-old child. Scale bar = 50 mm. **(C):** Hematoxylin and eosin-stained frozen sections of the corresponding proliferating hemangioma. **(D):** Hematoxylin and eosin-stained frozen sections of the corresponding involuted hemangioma. Scale bar = 50 μm.

Adherent cells isolated from hemangiomas were visible microscopically 12 hours after plating. Similar to MSCs derived from human BM [18], these Hem-MSCs grew into a fibroblast-like morphology and formed colonies when plated at low density. The cultured Hem-MSCs were homogeneous at passage 1, as determined by the cell surface antigenic profile that was analyzed by flow cytometry and indirect immunofluorescence staining (Fig. 2). The adherent MSCs from hemangiomas were uniformly positive for mesenchymal markers SH2 or CD105 (also known as endoglin), SH3, SH4, CD90, and the β-1 integrin CD29 and were negative for hematopoietic antigens CD45, CD14, c-kit, T-cell markers CD4 and CD8, endothelial markers CD31 and KDR, the hematopoietic/endothelial marker CD34, and the adipocyte-specific protein adiponectin. Some cells expressed α-SMA (Fig. 2B) but not calponin, a marker of more fully differentiated smooth muscle cells. The human stem/progenitor cell marker CD133 (Fig. 2A) was found only on early passage cells. CD133 is known to be quickly downregulated in culture [30, 31]. Hem-MSCs from proliferating and involuting hemangioma showed similar antigen expression profile.

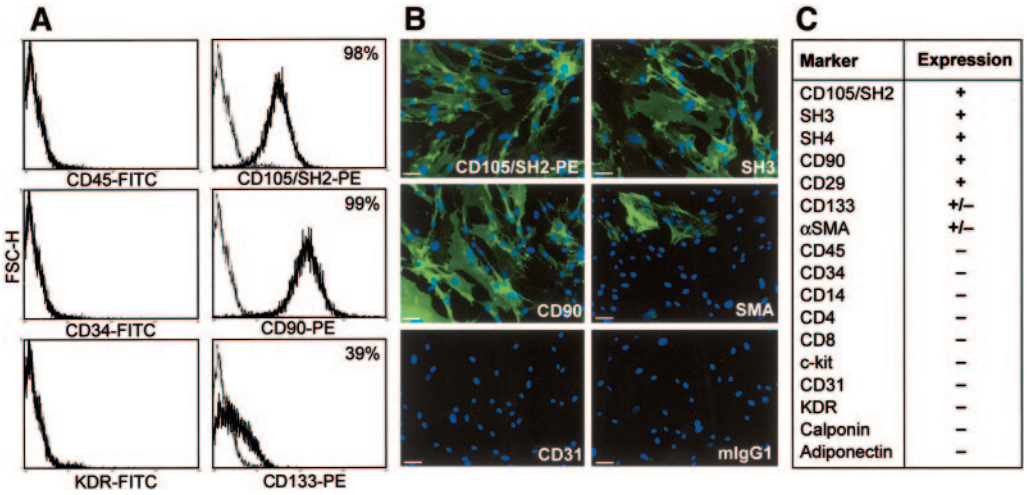


Figure 2. Cell surface antigen expression on Hem-MSCs. **(A):** MSCs isolated from infantile hemangioma were analyzed by flow cytometry. The grey line in the histogram represents cells labeled with FITC- or PE-conjugated immunoglobulin G isotype-matched control antibodies. The black line represents cells stained with FITC- or PE-conjugated antibodies. **(B):** MSCs isolated from infantile hemangioma were analyzed by indirect immunofluorescent staining. Positive antigen expression is seen as green fluorescence, and cell nuclei are seen as blue fluorescence. **(C):** The results of all the antigens examined are summarized. Hem-MSCs at passage 1 were homogeneously positive for SH2, SH3, SH4, CD90, and CD29 and negative for CD14, CD34, CD45, CD4, CD8, c-kit, CD31, KDR, calponin, and adiponectin. A subset of Hem-MSCs were positive for CD133 and α-SMA. Scale bar = 50 μm. Abbreviations: α-SMA, smooth muscle α-actin; FITC, fluorescein isothiocyanate; Hem-MSC, hemangioma-derived mesenchymal stem cell; KDR, kinase insert domain receptor; MSC, mesenchymal stem cell; PE, phycoerythrin.

In parallel studies, mesenchymal cells were isolated from five different human neonatal foreskins (F) using the same procedure (supplemental online Table 1). These cells exhibited morphology and immunophenotye similar to that of MSCs isolated from hemangioma (data not shown) but very little differentiation potential (Fig. 3Bd–f).

Multilineage Differentiation of Hem-MSCs

There are, as of yet, no known MSC-specific markers. Rigorous identification of MSCs requires demonstration of their capability to differentiate along specific mesenchymal lineages when induced to do so. Adipogenic differentiation of expanded Hem-MSCs was induced using the same culture conditions that have been employed for human BM MSCs [18]. Cytoplasmic lipid vacuoles labeled with Oil Red O were apparent on day 8 and continued to accumulate through day 16 (Fig. 3A). Hem-MSCs also differentiated into osteoblastic and myoblastic cells. von Kossa staining revealed mineralization of extracellular matrix produced by cultured Hem-MSCs (Fig. 3Bb). Calcium accumulation was evident after 1 week and increased over time. Multinucleated myoblasts were identified by positive immunostaining for skeletal muscle myosin (Fig. 3Bc). Parallel induction studies of mesenchymal cells isolated by the same procedure from infant skin showed some adipogenic differentiation, no calcium deposition, and no myotubes after 16 days (Fig. 3Bd, e, and f, respectively).

Hem-MSCs expanded after as many as 10 passages maintained their differentiation capacity, indicating their phenotypic stability and ability to undergo self-renewal in vitro. As shown in the supplemental online data, the numbers of adipogenic cells at passage 6 (P6) remained unaltered compared with passage 1 (P1). However, lipid accumulation was not as robust at P6 as that seen in P1 Hem-MSCs. By passage 10, both the numbers of adipogenic cells and lipid production were reduced (supplemental online Fig. 1). Decreased differentiation potential and senescence of MSCs in long-term culture has been described in MSCs derived from human BM [18, 32].

Adipogenic Propensity of MSCs in Proliferating Hemangioma

To determine whether Hem-MSCs isolated from proliferating and involuting hemangiomas behave differently in vitro, we compared cells derived from individual tumors in proliferation and differentiation assays. MSC isolates designated as Hem-60, 61, 63, and 64 from proliferating hemangiomas and Hem-I-29, -I-30, and -I-32 from involuting hemangiomas were plated at the same density and grown under identical conditions. Cell numbers were determined on days 0, 3, 6, and 10 (Fig. 4). During this time course, MSCs from the two phases grew at comparable rates, indicating no substantial differences in the in vitro growth potential of Hem-MSCs from proliferating or involuting hemangiomas.

In contrast, the number of adipocytes induced in Hem-MSCs from proliferating hemangiomas (n = 5) was significantly higher as compared with Hem-MSCs from involuting hemangiomas (n = 5) or from specimens of normal infant foreskin (n = 5) (Fig. 5). Adipogenic cells were identified by Oil Red O staining, counted, and expressed as a fraction of total cells, which was determined by counting cell nuclei counterstained with Gill's hematoxylin (Fig 5A). For comparison, Oil Red O staining of MSCs from one proliferating and one involuting hemangioma is shown in Figure 5B. These results were validated by relative quantitative reverse transcription-PCR analysis of *PPAR-γ-2*, a key regulatory gene of adipogenesis [33] (Fig. 5C). No differences were found in the osteogenic differentiation potential of Hem-MSCs from proliferating or involuting hemangiomas, as determined using the von Kossa histological stain for calcium deposition (Fig. 5D).

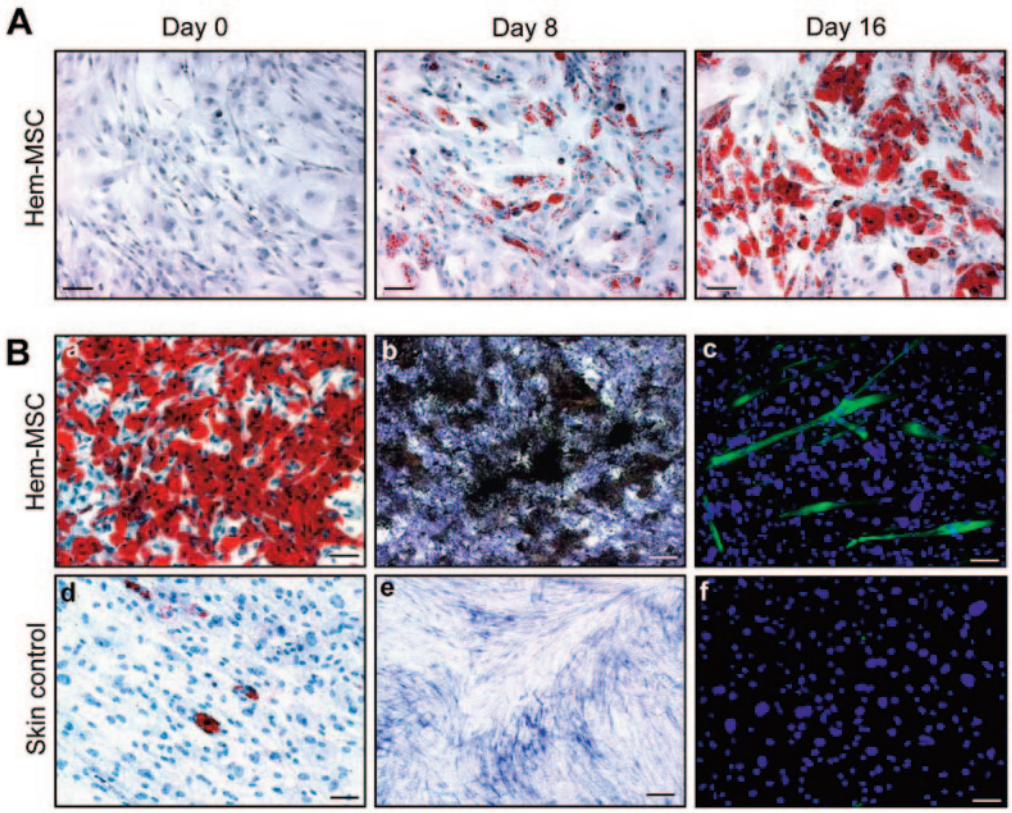


Figure 3. Differentiation of hemangioma-derived mesenchymal stem cells (Hem-MSCs). **(A):** Hem-MSCs differentiated into adipocytes in a time-dependent manner when incubated in adipogenic medium. Cells were stained with Oil Red O and counterstained with Gill's hematoxlin at days 0, 8, and 16 after induction. Cytoplasmic lipid vacuoles stain red with Oil Red O. Representative images of differentiated Hem-MSCs from a hemangioma designated 63 are shown. Scale bar = 50 μm. **(B):** Hem-MSCs (a–c) were induced to differentiate along adipogenic, osteogenic, and myogenic lineages. MSCs isolated from normal infant skin (d–f) were induced in parallel for comparison. Adipogenic differentiation is indicated by the accumulation of neutral lipid vacuoles stained red with Oil Red O (a, d). Osteogenic differentiation is shown by calcium deposition in black-brown (b), which is not seen in (e). Myogenesis is indicated by multinucleated myotubes stained with anti-skeletal muscle myosin in green (c), which is absent in (f). Representative images of differentiated Hem-MSCs from hemangioma 65 are shown. Scale bars = 50 μm (a, c, d, f), 250 μm (b, e).

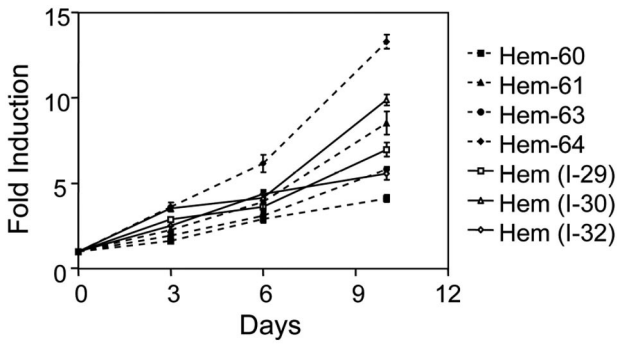


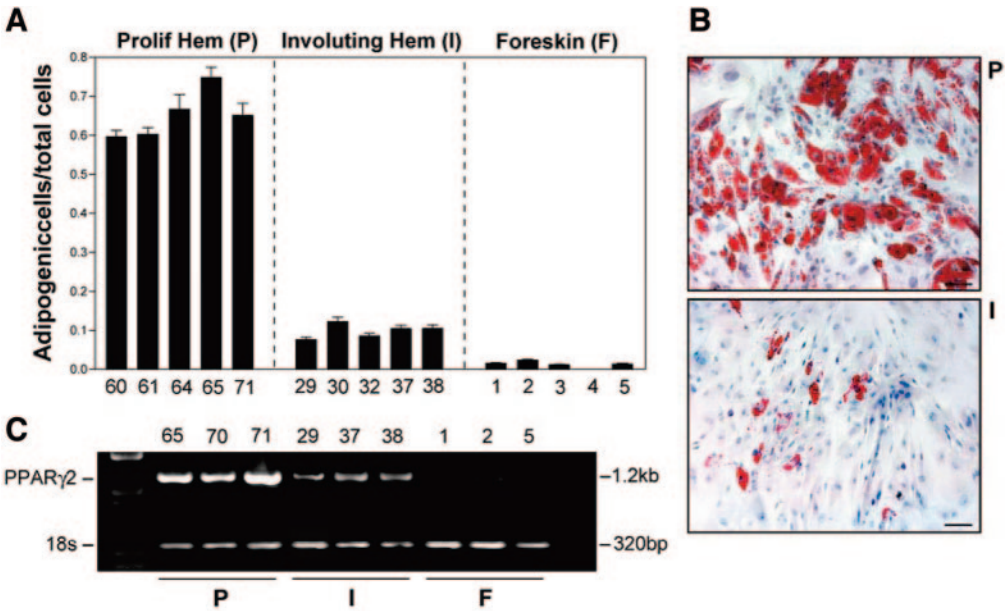
Figure 4. Proliferation of hemangioma-derived mesenchymal stem cells (Hem-MSCs). Hem-MSCs derived from proliferating hemangioma designated 60, 61, 63, and 64 (dashed lines) and involuting hemangioma I-29, I-30, and I-32 (solid lines) were plated at a density of 1×10^4 cells per well into 24-well tissue culture plates in the culture medium as described in Materials and Methods. Cell numbers were counted at days 0, 3, 6, and 10 and plotted as fold induction. The results are presented as the mean ± SD of quadruplicate determinations.

Clonality of Hem-MSCs

We have previously shown that endothelial cells derived from proliferating hemangioma (Hem-ECs) are clonal [8], suggesting that Hem-ECs arise from a single endothelial cell. The clonality and abnormal behavior of Hem-ECs suggest that hemangioma is caused by a somatic mutation(s) in a gene(s) regulating endothelial cell proliferation and/or differentiation [8, 9]. To examine the question of clonality of Hem-MSCs, MSCs from hemangiomas were analyzed for heterozygosity in the androgen receptor gene and if heterozygous, subjected to HUMARA assay to determine whether there was a nonrandom pattern of X-chromosome inactivation. As shown in Figure 6, both alleles were detected at approximately equal levels before and after digestion with the methylation-sensitive restriction enzyme Hha I in Hem-MSCs from four different hemangiomas. The clonal control sample of leiomyomata (Con) showed only one allele after HhaI digestion. This demonstrated that a random pattern of X-chromosome inactivation existed in the Hem-MSCs, indicating the cells were not clonal.

Discussion

The cellular constituents of hemangioma change throughout its life cycle of growth and regression. Endothelial cells that predominate in proliferating phase gradually decrease throughout involution. Adipocytes increase and eventually dominate in involuted phase. This accumulation of adipocytes was also



D Osteogenic differentiation of Hem-MSCs

MSCs	von Kossa Staining					
Proliferating Hem ($n = 6$)	+	++	+	+++	+++	++
Involuting Hem ($n = 6$)	+++	++	+	+++	++	++
Infant Skin ($n = 4$)	−	−	−	−		

Figure 5. Adipogenic propensity of Hem-MSCs derived from proliferating hemangioma. **(A):** Adipogenic differentiation was induced for 16 days in Hem-MSCs derived from proliferating hemangioma (P) 60, 61, 64, 65, and 71, involuting (I) hemangioma 29, 30, 32, 37, and 38, and in MSCs from infant skin 1–5. The numbers of adipogenic cells and total cells were quantitated as described in Figure 4. **(B):** Representative images of adipogenesis in Hem-MSCs from proliferating (P) and involuting (I) hemangioma. Scale bar = 50 mm. **(C):** Adipogenesis was further validated by relative quantitative RT-PCR analysis of PPAR-γ-2. Sample mRNA level was quantitated by 18s ribosomal RNA as an internal control. **(D):** MSCs derived from six proliferating hemangiomas, six involuting hemangiomas, and four neonatal foreskin controls were differentiated into osteogenic cell lineage. After 16-day induction, calcium deposition was quantified by von Kossa staining and rated as strong (+++), moderate (++), weak (+), or absent (−). Abbreviations: Hem, hemangioma; Hem-MSC, hemangioma-derived mesenchymal stem cell; MSC, mesenchymal stem cell; PPAR, peroxisome proliferator-activated receptor; RT-PCR, reverse transcription-polymerase chain reaction.

reflected by gene expression patterns in involuting hemangioma. Using cDNA microarray, several genes found to be overexpressed in the involuting hemangioma are involved in lipid metabolism [34]. These include the adiponectin gene (*APM1*), perilipin, fatty acid binding protein, and lipoprotein lipase (data not shown). We hypothesize that MSCs are the source of adipocytes that appear during involution. In this study, we demonstrate that MSCs are present in infantile hemangioma, and these cells showed significant adipogenic potential when induced under the appropriate conditions in vitro.

MSCs derived from proliferating and involuting hemangiomas exhibited fibroblast-like morphology and typical MSC-like immunophenotype [18]. Flow cytometry showed that the cell surface antigens CD45, CD14, CD34, CD31, and KDR were not detectable on these cells, indicating that Hem-MSCs were not contaminated with cells of hematopoietic or endothelial origin. More importantly, Hem-MSCs exhibited self-renewal capacity and ability to differentiate into multiple mesenchymal cell lineages: adipocytes, osteoblasts, and skeletal myoblasts. In contrast, MSCs were not isolated from PG, a distinct endothelial proliferative lesion that can occur in children. Only limited numbers of mesenchymal cells isolated from normal neonatal foreskins showed capacity to differentiate into adipocytes. We and others have shown that MSCs can be isolated from human skin and exhibit multilineage differentiation ability [35, 36]. It is possible that the number of MSCs derived from foreskin was too low to detect osteogenic and myogenic cells under the conditions and culture times used in this study. Our data here strongly support the presence of MSCs in both proliferating and involuting hemangioma.

The decreased differentiation capacity of Hem-MSCs by passage 10 is similar to decreased differentiation potential and senescence previously described in MSCs derived from human BM [18, 32]. Recent evidence suggests that this finite life span of cultured human MSCs is due to lack of telomerase activity [37]. Despite a growing body of information regarding MSCs, the mechanisms that govern MSC self-renewal and multilineage differentiation remain poorly understood.

Hem-MSCs derived from proliferating and involuting hemangiomas behaved similarly in vitro in terms of proliferation and osteogenic differentiation. But when induced to differentiate into adipocytes, the number of adipogenic Hem-MSCs from proliferating hemangiomas was significantly higher than from involuting tumors or from normal infant foreskin. This difference was quantified by counting the number of Oil Red O-positive cells and the total number of cell nuclei in the cultures.

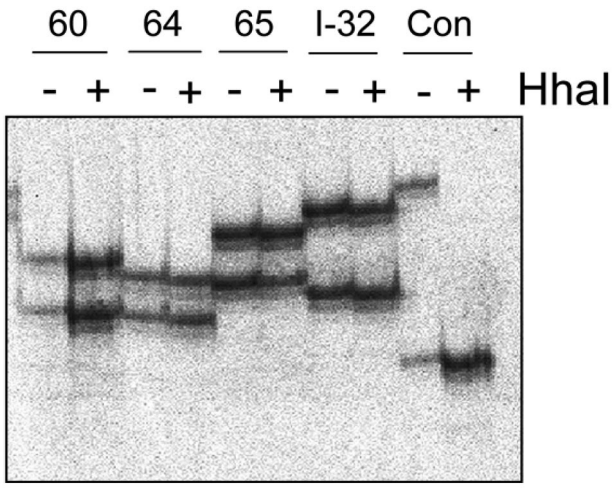


Figure 6. Clonality of Hem-MSCs. Genomic DNA from Hem-MSCs from hemangioma 60, 54, 65, and I-32 and a positive clonal control from a leiomyomata tissue sample were subjected to HUMARA assay. Note that both alleles were detected at equal levels before and after digestion with the methylation-sensitive restriction enzyme Hha I in Hem-MSCs, indicating that Hem-MSCs are not clonal. Abbreviations: Hem-MSC, hemangioma-derived mesenchymal stem cell; HUMARA, human androgen receptor.

The levels of PPAR-γ-2 mRNA expression validated this adipocyte quantitation. In contrast, the levels of another isoform, PPAR-γ-1, were constant and did not change in cells from proliferating and involuting hemangioma or from normal foreskin (data not shown). The results are consistent with the concept that expression of PPAR-γ-2, but not PPAR-γ-1, correlates with the degree of lipid accumulation [33]. The decrease in the number of adipogenic Hem-MSCs from the involuting hemangioma could be because MSCs had already differentiated into adipocytes and, hence, could not be recovered in our isolation procedure. Another possibility is that MSCs are recruited to the proliferating tumor from BM or localized tissue niches of MSCs, but once involution begins the recruiting signals diminish.

We have shown that Hem-ECs are clonal and exhibit abnormal behavior in vitro [8, 10, 11]. This suggests that hemangioma arises from a single progenitor cell, and hemangioma may be caused by somatic mutation(s) in genes regulating EC proliferation and/or differentiation. Here, we show that Hem-MSCs are not clonal, as determined by the X-chromosome inactivation of androgen receptor gene (HUMARA assay). This indicates that Hem-MSCs are expanded from a population of MSCs to become a constituent of the stromal compartment of hemangioma.

The origin of Hem-MSCs is unclear. It is known that human MSCs can be obtained from a variety of sources, including BM, blood, dermis, bone, and adipose tissue [17]. Gene profiling has shown that the expression patterns of cord blood-derived MSCs are similar to those of BM-derived MSCs [29, 38]. Transplantation of human MSCs in fetal sheep and blastocytes of mice and nonhuman primates have resulted in engraftment and site-specific differentiation in various tissues [22–26]. Evidence has also emerged from animal studies showing preferred engraftment of transplanted MSCs and concomitant regeneration in diseased tissues such as muscles in mice with muscular dystrophy [39], cutaneous wounded in rats [40], and melanomas in mice [41]. Thus, it is reasonable to postulate that Hem-MSCs are recruited to the site of hemangioma from BM through the circulation or from local or nearby MSC niches in the dermis and adipose tissue. Further experiments to study Hem-EC-MSC interactions with other cellular constituents within the tumor and to identify hemangioma-derived factors that trigger mobilization, localization, and differentiation of MSCs in hemangioma will contribute to our understanding of the mechanism of involution.

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DISCLOSURES

The authors indicate no potential conflicts of interest.

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