

Brief report

Endothelial progenitor cells in infantile hemangioma

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Infantile hemangioma is an endothelial tumor that grows rapidly after birth but slowly regresses during early childhood. Initial proliferation of hemangioma is characterized by clonal expansion of endothelial cells (ECs) and neovascularization. Here, we demonstrated mRNA encoding CD133-2, an important marker for endothelial progenitor cells (EPCs), predominantly in proliferating but not involuting

or involuted hemangioma. Progenitor cells coexpressing CD133 and CD34 were detected by flow cytometry in 11 of 12 proliferating hemangioma specimens from children 3 to 24 months of age. Furthermore, in 4 proliferating hemangiomas, we showed that 0.14% to 1.6% of CD45⁻ nucleated cells were EPCs that coexpressed CD133 and the EC marker KDR. This finding is consistent with the

presence of KDR⁺ immature ECs in proliferating hemangioma. Our results suggest that EPCs contribute to the early growth of hemangioma. To our knowledge, this is the first study to show direct evidence of EPCs in a human vascular tumor. (Blood. 2004;103:1373-1375)

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Introduction

Although hemangioma is the most common tumor of infancy,¹ its cause remains unknown. The life span of infantile hemangioma is generally divided into proliferating phase (0-1 year), involuting phase (1-5 years), and involuted phase (5-10 years).^{2,3} Early proliferating hemangioma is composed of densely packed endothelial cells (ECs). These ECs have been described as "angioblastic" and shown to be more embryonic than neonatal microvascular ECs based on morphology and protein expression patterns.⁴⁻⁸ These findings suggest that hemangioma contains primitive ECs. Recently, we and others showed that hemangioma-derived ECs are clonal and exhibit abnormal behavior,^{9,10} suggesting hemangioma arises from clonal expansion of a single EC carrying a somatic mutation. We hypothesize that endothelial progenitor cells (EPCs) play a crucial role in the hemangiogenesis, perhaps as precursors of the clonal ECs. The aim of this study was to determine whether EPCs are present in hemangioma.

EPCs have been found in bone marrow, blood circulation, fetal liver, and skeletal muscle.^{11,12} Recent studies suggested that EPCs, hematopoietic stem cells (HSCs), and progenitor cells contribute to embryonic tissue vascularization, postnatal organ regeneration, and tumor neoangiogenesis.^{13,14} Identification of EPCs relies on specific cell-surface proteins. CD133, also called AC133 antigen and human prominin-1,¹⁵ is a novel human stem/progenitor cell marker. Endothelial markers including CD34, CD31, von Willebrand factor (VWF), and the VEGF KDR are expressed by EPCs, vascular wall-derived mature ECs, and subsets of hematopoietic cells, whereas CD133 is expressed only in progenitor cells.¹⁴ Hence, we used CD133 combined with endothelial markers for rigorous EPC identification.

In this study, we examined CD133 gene expression during hemangioma evolution by Northern blotting and reverse transcrip-

tion-polymerase chain reaction (RT-PCR). Using flow cytometry, we showed that proliferating hemangioma contains EPCs that coexpress CD133 and an endothelial marker KDR. These findings suggest that EPCs participate in hemangioma pathogenesis.

Study design

Patient samples

Cutaneous hemangiomas and healthy skin from age-matched donors were obtained with approval from the Committee on Clinical Investigation, Children's Hospital (Boston, MA). Clinical diagnosis of hemangiomas was confirmed by histologic and immunochemical analysis carried out by the Department of Pathology, Children's Hospital.

CD133 mRNA expression

A probe amplified from RNA of WERI-RB-1 retinoblastoma cells (American Type Culture Collection, Manassas, VA) by RT-PCR using primers 5'-CCAAGTTCTACCTCATGTTTGG-3' and 5'-ACCACAGGGAGAT-TGCAAAGC-3' was used to detect CD133 transcripts.¹⁶

Detection of EPCs

After excising the epidermis and washing off blood, hemangioma nodules were separated from connective tissue, minced, and digested with 1 mg/mL collagenase A (Roche, Indianapolis, IN) in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) at 37°C for 1 hour. Tissue was homogenized and filtered through 100- and 40- μ m cell strainers (Fisher Scientific, Pittsburgh, PA). Red blood cells were lysed in ammonium chloride (StemCell Technologies, Vancouver, BC, Canada). In 4 experiments, hematopoietic mononuclear cells were depleted with anti-CD45 magnetic microbeads (Miltenyi Biotec, Auburn, CA). Cells labeled

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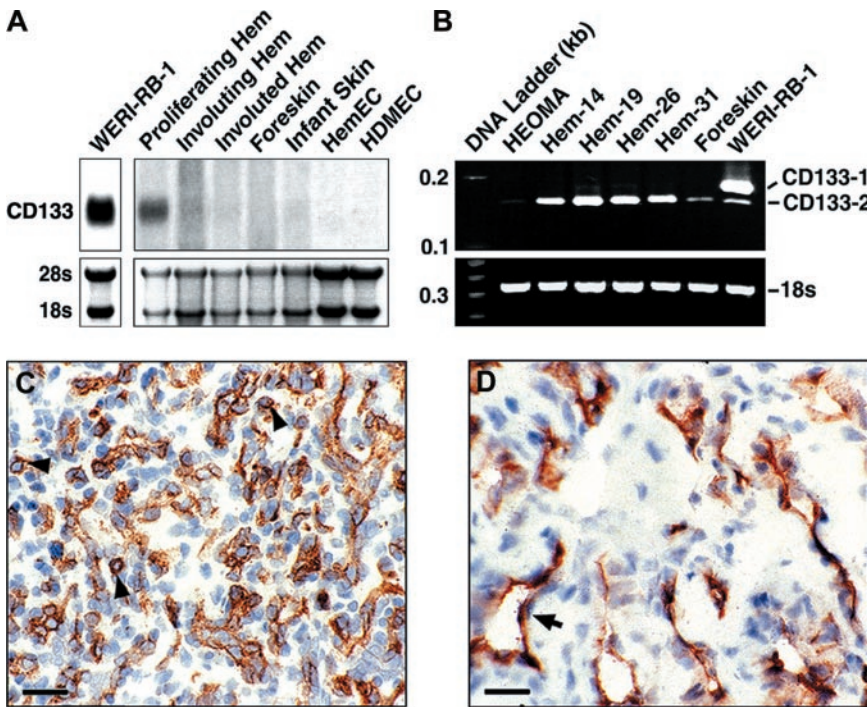


Figure 1. CD133-2 and KDR⁺ immature ECs in proliferating hemangioma. (A) Pooled RNA (5 μg) from proliferating, involuting, or involuted hemangioma tissues, neonatal foreskins, or healthy infant skins and 15 μg RNA from cultured WERI-RB-1, hemangioma-derived ECs (HemEC) or human dermal microvascular ECs (HDMECs) were analyzed by Northern blotting using a ³²P-labeled CD133 probe. The blot was exposed for 8 hours (upper left) and 10 days (upper right). RNA levels were verified by ethidium bromide-stained rRNA. (B) CD133-1 and CD133-2 were amplified by RT-PCR from hemangioendothelioma (HEOMA), proliferating hemangiomas designated 14, 19, 26, and 31 from patients 4 to 12 months of age, neonatal foreskin, and WERI-RB-1 retinoblastoma cells. Equivalent mRNA level was indicated by 18s rRNA. Frozen sections of proliferating (C) and involuting (D) hemangioma from patients 5 months and 2 years of age, respectively, were stained with an antihuman KDR mAb and developed with AEC chromagen. Cell nuclei were counterstained with hematoxylin. Interstitial KDR⁺ cells and examples of flat ECs are indicated by arrowheads and arrow, respectively. Scale bar is 20 μm.

with phycoerythrin (PE)-conjugated anti-CD133 (Miltenyi Biotech), fluorescein isothiocyanate (FITC)-conjugated anti-KDR, anti-CD34 (Miltenyi Biotech), or anti-CD45 (R&D Systems, Minneapolis, MN) were analyzed by flow cytometry.¹⁷ KDR monoclonal antibody (mAb; clone KDR-1, Sigma, St Louis, MO) was purified using protein G-agarose (Pierce, Rockford, IL), conjugated to fluorescein using the Alexa Fluor 488 monoclonal antibody labeling kit (Molecular Probes, Eugene, OR), and characterized by its binding to KDR-transfected PAE cells.

Results and discussion

CD133-2 and KDR in proliferating hemangioma

By Northern blotting, CD133 mRNA was detected in proliferating hemangioma but not involuting and involuted hemangioma, nor healthy skin and cultured ECs (Figure 1A). The size of CD133 transcript was the same as that in the positive control RNA of WERI-RB-1 cells. Proliferating hemangioma expressed predominantly CD133-2, a novel splice variant of CD133¹⁷ (Figure 1B). In

contrast, CD133-2 was barely detectable in a human hemangioendothelioma, an endothelial tumor that does not regress.

Localization of CD133 antigen on stem/progenitor cells in tissue sections has not been reported due to unavailability of an antibody suitable for immunohistochemical staining. However, in proliferating hemangioma, anti-KDR antibody recognized plump ECs with “immature” morphology, that is, large nuclei and scant cytoplasm, lining small nascent vessels but also in the interstitial regions (Figure 1C). In contrast, flattened KDR⁺ ECs were found on the more established vessels in involuting hemangioma (Figure 1D). The presence of “immature” ECs in proliferating hemangioma is consistent with the CD133 mRNA expression patterns.

CD133⁺CD34⁺ progenitor cells in proliferating hemangioma

We began analyzing progenitor cells in proliferating hemangioma at a time when conjugated anti-KDR antibody was not available. Using 2-color channel flow cytometry, we detected CD133⁺CD34⁺

Table 1. Quantification of CD133⁺CD34⁺ cells in proliferating hemangioma

	Hemangioma specimen	Patient sex	Patient age, mo	Tumor first noted	CD34 ⁺ , %	CD133 ⁺ CD34 ⁺ , %	CD133 ⁺ KDR ⁺ , %
1	38	F	7	2 wk after birth	45.2	0.18	ND
2	39	M	7	3 wk after birth	23.2	0.10	ND
3	41	F	4	At birth	27.2	21.40	ND
4	46	F	5	At birth	21.6	0.00	ND
5	49	F	15	1 wk after birth	34.3	0.15	ND
6	53	M	9	2 mo after birth	49.9	0.67	ND
7	54	F	19	8 d after birth	69.9	2.91	ND
8	61	F	3	2 wk after birth	53.4	1.62	ND
9	73	F	4	2–3 d after birth	50.3	0.20	0.17
10	74	F	6	At birth	34.6	0.17	0.14
11	75	F	15	2–3 d after birth	37.8	2.15	1.60
12	77	F	24	At birth	69.4	0.23	0.22

ND indicates not determined.

cells in 11 of 12 proliferating hemangiomas from patients 4 to 24 months of age (Table 1). They varied from 0.1% to 2.9% of total cells among 10 positive specimens with no correlation with patient age or tumor duration. This corresponds to 1×10^4 to 2.9×10^5 of CD133⁺CD34⁺ cells in a $1 \times 1 \times 1$ -cm³ size early proliferating tumor, which yields 1×10^7 cells on average. In specimen designated 41, remarkably, 21.4% of total cells were CD133⁺CD34⁺ cells. In contrast, no double-positive cells were detected in specimen 46, suggesting that EPC is not involved in all hemangiomas. CD133⁺CD34⁺ cells were also not found in 3 involuting hemangiomas from patients 5 months to 2 years of age and a hemangioendothelioma (data not shown), consistent with CD133 expression. The CD133⁺CD34⁺ cell population is most likely composed of EPCs and perhaps some HSCs derived from blood in the tumor tissue. It is worth noting that early proliferating hemangioma contains little blood due to lack of established vascular network.

EPCs in proliferating hemangioma

We further validated the presence of EPCs by labeling cells isolated from proliferating hemangioma with PE-conjugated anti-CD133 and FITC-conjugated anti-KDR. CD45⁺ mononuclear cells of hematopoietic origin were first removed with anti-CD45 magnetic microbeads. In specimens 73, 74, 75, and 77, 0.17%, 0.14%, 1.6%, and 0.22% of total cells, respectively, were EPCs coexpressing CD133 and KDR (Table 1). Detailed analysis of specimen 75 is shown in Figure 2. Interestingly, 0.5% of the cells expressed CD133 but neither KDR nor CD45, suggesting the presence of other types of stem/progenitor cells. No EPC was detected in 3 specimens of venous malformation and 2 of lymphatic malformation (data not shown), indicating a certain selectivity of EPCs in contributing to hemangioma pathogenesis.

The differences in EPC percentage among individual hemangiomas were not unexpected, given that the specimens were obtained at different ages based on clinical decisions for the best care of the children. These differences may reflect variations in the rate of tumor evolution or the stage of EPC differentiation. It is well known that there is histologic variability among hemangiomas from patients of the same age, as well as within microscopic regions in the same specimen.

The primary cause of hemangioma is unknown. Our previous studies support the hypothesis that hemangioma arises when somatic mutations occur in a single EC, leading to dysregulation of

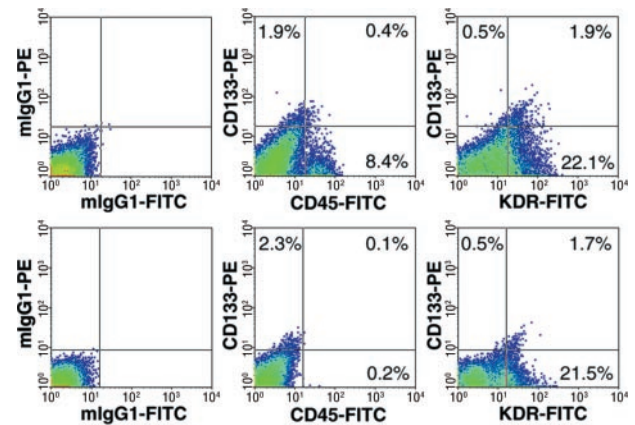


Figure 2. EPCs coexpressing CD133 and KDR in proliferating hemangiomas. Hematopoietic cells were removed from a single cell suspension prepared from proliferating hemangioma specimen 75 using anti-CD45 magnetic microbeads. Before (top) and after (bottom) depletion, cells were double-labeled with PE- or FITC-conjugated mouse antihuman isotype-matched IgG controls, or PE-conjugated anti-CD133 and FITC-conjugated anti-CD45, or PE-conjugated anti-CD133 and FITC-conjugated anti-KDR. Labeled cells were analyzed by flow cytometry and 20 000 events were acquired. Percentage of cell population expressing single or double markers are shown in each quadrant.

the genes that control EC proliferation and differentiation.^{9,16} Identification of EPCs raises the possibility that these cells may give rise to clonal ECs, and thereby initiate uncontrolled EC growth. On the other hand, we cannot exclude the possibility that EPCs are recruited later from elsewhere during angiogenesis of proliferating hemangioma. In vitro properties of hemangioma-derived EPCs are currently under investigation. Whether EPCs originate from bone marrow or a specific tissue niche for stem/progenitor cells remains to be determined.

In conclusion, we demonstrated that CD133⁺KDR⁺ EPCs are present in proliferating hemangioma. This finding suggests that EPCs contribute to early expansion of hemangioma. Further investigation is needed to determine the precise pathogenic roles and potential therapeutic implications of these EPCs.

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