Title: Endothelial Progenitor Cells in Infantile Hemangioma

Short Title: Endothelial Progenitors in Hemangioma

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Abstract

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 co-expressing CD133 and CD34 were detected by flow cytometry in 11/12 proliferating hemangioma specimens from children 3 to 24 months of age. Furthermore, in 4 proliferating hemangiomas, we showed that 0.14 - 1.6% of CD45negative nucleated cells were EPCs that co-expressed CD133 and the endothelial cell 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.
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 year) and involuted phase (5-10 year)\(^2,3\). Early proliferating hemangioma is composed of densely-packed 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 EPCs play a crucial role in the hemangiomagenesis, 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, vWF, and KDR are expressed by EPCs, vascular wall-derived mature ECs and subsets of
hematopoietic cells, whereas CD133 is only expressed in progenitor cells\textsuperscript{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 RT-PCR. Using flow cytometry, we showed that proliferating hemangioma contains EPCs that co-express CD133 and an endothelial marker KDR. These findings suggest that EPCs participate in hemangioma pathogenesis.
Study design

Patient samples

Cutaneous hemangiomas and normal skin from age-matched donors were obtained with approval from the Committee on Clinical Investigation, Children’s Hospital Boston. Clinical diagnosis of hemangiomas was confirmed by histological and immunohistochemical analysis carried by the Department of Pathology, Children’s Hospital Boston.

CD133 mRNA expression

A probe amplified from RNA of WERI-RB-1 retinoblastoma cells (ATCC, Manassas, VA) by RT-PCR using primers 5’-CCAAGTTCTACCTCATGTGGATG-3’ and 5’-ACCACCAGGAGATTGCAAAAGC-3’ was used to detect CD133 transcripts.

EPCs detection

After excising the epidermis and washing off blood, hemangioma nodules were separated from connective tissue, minced, and digested with 1 mg/ml of collagenase A (Roche, Indianapolis, IN) in PBS containing 0.1% BSA at 37ºC for 1 hour. Tissue was homogenized and filtered through 100 and 40 μM cell strainers (Fisher Scientific, Pittsburgh, PA). Red blood cells were lysed in ammonium chloride (StemCell Technologies, Vancouver, Canada). In four experiments, hematopoietic mononuclear cells were depleted with anti-CD45 magnetic microbeads (Miltenyi Biotech, Auburn, CA). Cells labeled with PE-conjugated anti-CD133 (Miltenyi Biotech), FITC-conjugated anti-KDR, anti-CD34 (Miltenyi Biotech), or anti-CD45 (R & D Systems, Minneapolis, Minnesota).
MN) were analyzed by flow cytometry\textsuperscript{17}. KDR mAb (Sigma, clone KDR-1, St. Lois, MO) was purified using Protein G-agarose (PIERCE, Rockford, IL), conjugated to fluorescein using 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 normal skin and cultured ECs (Fig. 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 CD133$^{17}$ (Fig. 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, i.e., large nuclei and scant cytoplasm, lining small nascent vessels but also in the interstitial regions (Fig. 1C). In contrast, flattened KDR$^+$ ECs were found on the more established vessels in involuting hemangioma (Fig. 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$^+$ cells in 11/12 proliferating hemangiomas from patients 4 to 24 months of age (Table 1). They varied from 0.1 - 2.9% of total cells among 10 positive
specimens with no correlation with patient age or tumor duration. This corresponds to $1 \times 10^4 - 2.9 \times 10^5$ of CD133$^+$CD34$^+$ cells in a $1 \times 1 \times 1 \text{cm}^3$ size early proliferating tumor, which yields $1 \times 10^7$ 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 were EPCs co-expressing CD133 and KDR (Table 1). Detailed analysis of specimen 75 is shown in Fig. 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 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 histological variability among hemangiomas from patients of 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 the genes that control EC proliferation and differentiation. 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.
Acknowledgement

We thank Dr. Lawrence F. Brown at Beth Israel Deaconess Medical Center for his help with \textit{in situ} hybridization to detect CD133.
References


Table 1. Quantification of CD133+CD34+ Cells in Proliferating Hemangioma

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F, female. M, male. w, week. m, months. d, day.
Figure Legends

Fig. 1. CD133-2 and KDR+ immature ECs in proliferating hemangioma. (A) 5 µg pooled RNA from proliferating, involuting, involuted hemangioma tissues, foreskins or normal skins and 15 µg RNA of cultured WERI-RB-1, hemangioma-derived ECs (HemEC) or human dermal microvascular ECs (HDMEC) were analyzed by northern blotting using a \(^{32}\)P-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 ribosomal RNA. (B) CD133-1 and -2 were amplified by RT-PCR from hemangioendothelioma (HEOMA), proliferating hemangiomas designated 14, 19, 26 and 31 from patients 4-12m 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 5m and 2y of age, respectively, were stained with an anti-human KDR mAb and developed with AEC chromagen. Cell nuclei were counter-stained with hematoxylin. Interstitial KDR+ cells and examples of flat ECs are indicated by arrow heads and arrow. Scale bar, 20 µm.

Fig. 2. EPCs co-expressing 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 anti-human 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.
Figures

Figure 1
Figure 2
Endothelial Progenitor Cells in Infantile Hemangioma

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