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Haemogenic endothelium in infantile haemangioma

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ABSTRACT

Background Proliferating infantile haemangioma (IH) is a tumour of the microvasculature composed predominantly of immature endothelial cells. The origin of IH is unclear, but it has been shown to express markers of both endothelial and haematopoietic lineages, and a role for endothelial progenitor cells in the aetiology of IH has been suggested. Haemangioblasts are precursors of both endothelial and haematopoietic cells, and their characterisation has identified the expression of cell surface and intracellular proteins that collectively can be used for assigning a haemangioblast phenotype.

Methods The authors used immunohistochemical staining to characterise the expression of primitive haematopoietic-associated proteins in proliferating IHs.

Results and discussion The authors show that the cells forming the capillary endothelium express markers associated with primitive haematopoietic cells. Additionally, many of these cells express the transcription factors brachyury and GATA-2, indicating a primitive mesodermal origin. They hypothesise that the immature capillaries in IH are derived from primitive mesodermal cells with haemangioblastic differentiation capabilities. The expression of primitive mesodermal, endothelial and haematopoietic markers by the cells forming the endothelium suggests that the immature capillaries that predominate in proliferating IH are a haemogenic endothelium phenotype, derived from haemangioblasts.

INTRODUCTION

Infantile haemangioma (IH), a primary tumour of the microvasculature, is the most common tumour of infancy, affecting about 10% of white children. It typically undergoes postnatal growth during infancy (proliferating phase) characterised by excessive angiogenesis, followed by spontaneous involution over the next 1–5 years (involuting phase) with continued fibro-fatty deposition that replaces the cellular elements up to 10 years of age (involved phase).^{1–3} Proliferating IH is composed predominantly of immature endothelial cells¹ with a smaller component of myeloid haematopoietic cells^{4–5} and mesenchymal stem cells.⁶ The lesion consists of immature capillaries with tiny lumens lined by plump endothelial cells with an outer concentric pericyte layer, characterised by its immunoreactivity (IR) for smooth muscle actin (SMA).^{1–2}

Endothelial progenitor cells (EPCs) have been termed haematopoietic stem cells with an innate ability to differentiate into mature endothelial cells.⁷ There is increasing evidence of the presence of an EPC population characterised by the expression of surface antigens CD133, CD34 and vascular endothelial growth factor receptor-2 (VEGFR-2)⁸

within proliferating IH, with a significant increase in this population of cells in the peripheral blood of affected patients.⁹ These EPCs have been presumed to be recruited into proliferating IH and contribute to the formation of the endothelial component of this tumour.⁸ We have recently shown that the expression of these EPC-associated markers is localised to the endothelium of the immature capillaries in proliferating IH¹⁰ and that these same capillaries have a primitive mesoderm neural crest phenotype.¹¹

Haemangioblasts are precursors of both haematopoietic and endothelial cells that have been shown to express brachyury,¹² TAL-1,¹² VEGFR-2,¹² GATA-2,¹³ CD31,¹³ ACE¹⁴ and CD34.¹³ The expression of brachyury,¹⁵ TAL-1,¹⁵ GATA-2¹⁶ and ACE¹⁴ on a subset of CD34+/VEGFR-2+ cells has been proposed to delineate primitive precursors from downstream EPCs. Previous reports describing the expression of both haematopoietic and endothelial markers in IH led us to investigate, in this study, whether cells expressing markers consistent with being haemangioblasts¹⁷ with the potential to differentiate to cells of both the haematopoietic and endothelial lineages, were present within IH.

METHODS

Biopsies of proliferating IH affecting the skin and/or subcutaneous tissues (figure 1A) from 12 patients, aged 3–10 (mean 6) months, were obtained according to a protocol approved by the Wellington Regional Ethics Committee. The tissues were formalin-fixed and paraffin-embedded using standard procedures. H&E and immunohistochemical (IHC) staining were performed on all 4 µm paraffin embedded serial sections. Routine rehydration followed by antigen retrieval with boiling 10 mM sodium citrate (Sigma-Aldrich, St Louis, Missouri) was performed, and sections were washed in Tris-buffered saline (TBS; 20 mM Tris HCl buffer pH 7.6 containing 135 mM NaCl) containing 0.1% Tween 20 (Sigma-Aldrich). Auto-fluorescence was quenched with 0.5% sodium borohydride (Sigma-Aldrich) prepared in TBS, and the slides then blocked with 5% bovine serum albumin (Sigma-Aldrich) prepared in TBS containing 0.1% Tween 20.

Immunolabelling with primary antibodies specific for antihuman brachyury, VEGFR-2, CD34, CD45, ACE, GATA-2, CD133, TAL-1, CD34, CD31, SMA and GLUT-1 (Abcam, Cambridge, Massachusetts) were performed overnight. Bound antibodies were visualised by incubation with the appropriate species-specific fluorochrome-conjugated secondary antibody (goat antimouse Alexa-fluor 488 or chicken antirabbit Alexafluor 594 (Invitrogen, Auckland, NZ)) for 2 h, with the exception of VEGFR-2 and GATA-2 which were

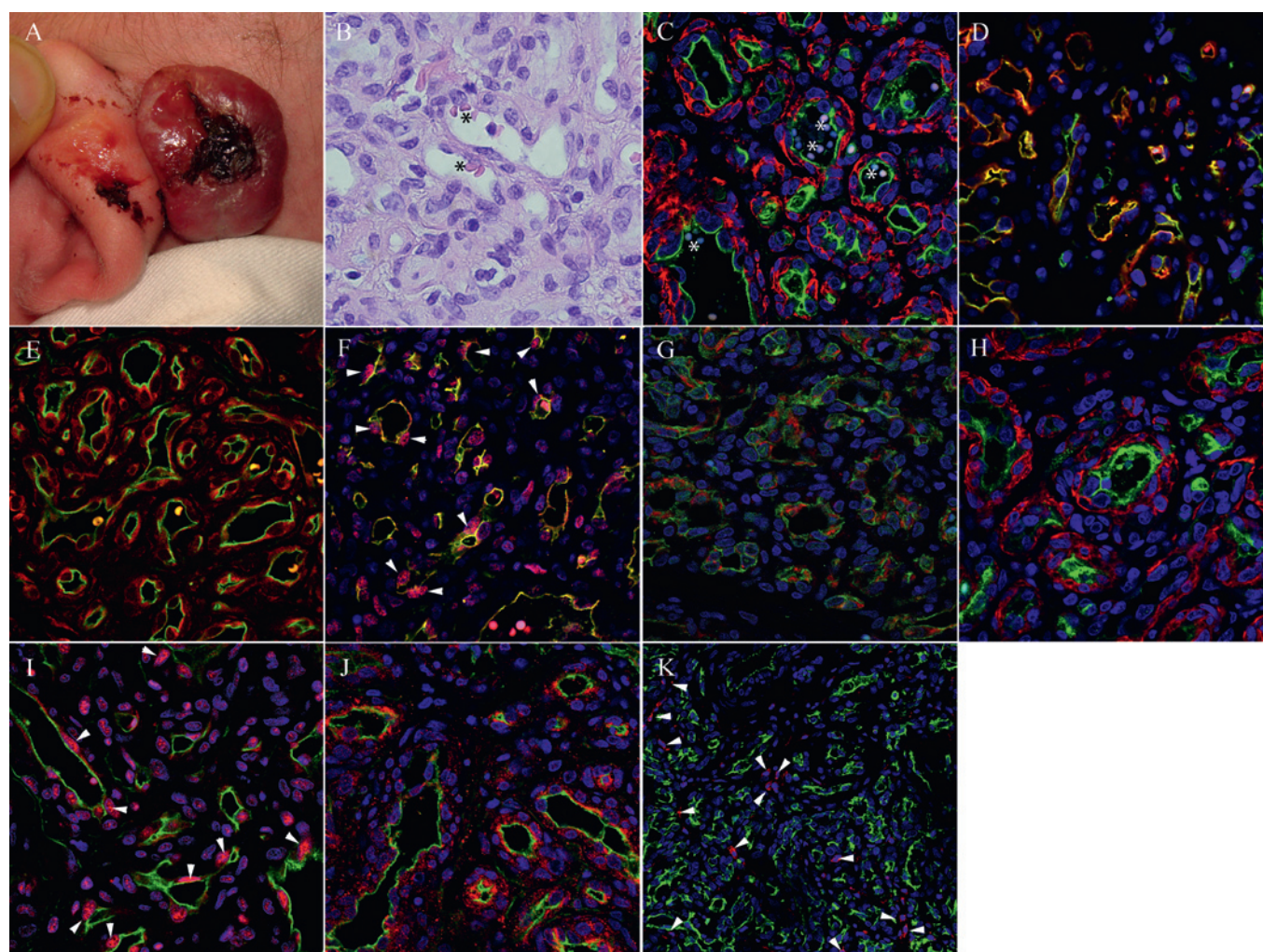


Figure 1 (A) Ulcerated proliferating IH on the left mastoid region in a 4-month-old boy. (B) H&E section of this lesion showing immature capillaries with a small lumen lined by plump endothelial cells. Erythrocytes can be seen within the lumen of some, but not all, of the microvessels (*). (C) Endothelium of the immature capillaries, readily identified and distinguished from the pericyte layer by the expression of CD34 (green). The pericyte layer, but not the endothelial cell layer, expresses smooth muscle actin (SMA) (red). (D) Staining for GLUT-1 (red) and CD34 (green). The endothelial cell layer (staining green), but not the pericyte layer, expresses GLUT-1. (E) CD34⁺ cells (green) that form the endothelium are also immunoreactive (IR) for CD133 (red). (F) CD34⁺ cells (green) also express vascular endothelial growth factor receptor 2 (red). (G) Endothelial cells forming the endothelium that express CD31 (green) and are also IR for brachyury (red). (H) Staining for SMA (red) identifying the pericyte layer, and ACE (green) which is confined to the endothelium. ACE is expressed only by the cells of the endothelium and not by the pericytes. (I) CD34⁺ cells (green), also express the haematopoietic transcription factor TAL-1 (red). Selected cells that show strong nuclear expression of TAL-1 are identified by white arrowheads. (J) Staining for CD34 (green) and GATA-2 (red). GATA-2 is expressed by the CD34⁺ cells. (K) CD31⁺ endothelial cells (green) forming the endothelium do not stain for the mature haematopoietic marker CD45 (red). CD45 IR is restricted to scattered cells within the interstitium (white arrowheads). All images are counterstained with 4',6-diamidino-2-phenylindole (blue).

detected with an antirabbit digoxigenin (Roche diagnostics, Auckland, NZ) conjugate followed by an antidigoxigenin-Rhodamine (Chemicon, Sydney, Australia) conjugate. Slides were mounted in Antifade Gold (Invitrogen) containing 4',6-diamidino-2-phenylindole as counterstain prior to visualisation. Paraffin sections of placental tissue were used as positive controls for CD133,¹⁸ CD34,¹⁹ ACE²⁰ and GATA-2.²¹ Negative controls included staining of paraffin sections of tissue not expected to be immunoreactive with the same series of antibodies (human uterine fibroid), and also by omission of the primary antibodies when staining selected proliferating IHs. These controls showed the expected staining pattern consistent with the previous reported specificity for the antibodies used. Images were captured using a Leica TCS 4D confocal laser-scanning microscope fitted with a krypton/argon laser (Leica Lasertechnik, Heidelberg, Germany).

RESULTS

Endothelium of proliferating IH expresses endothelial, haematopoietic and primitive mesodermal markers

Proliferating IH tissue taken from all 12 patients in this study showed typical characteristics on H&E sections (figure 1B). Erythrocytes can be seen within the lumen of some (*) but not all microvessels. The microvessels are composed of an inner cell layer that lines the endothelium and an outer concentric pericyte layer. Figure 1C clearly shows that the cells forming the endothelium are strongly immunoreactive (IR) for CD34 (green), whereas the cells that make up the outer pericyte layer shows IR for SMA (red), as expected. The CD34 IR is restricted to the lumen face of the cells forming the endothelium as has been shown in other studies.²² Autofluorescent erythrocytes can also be seen in some vessels (*). GLUT-1 is an IHC marker used to distinguish IH from other vascular anomalies.³ All samples

analysed showed IR for GLUT-1 (figure 1D, red) on the cells lining the endothelium. The GLUT-1 IR is predominantly localised to the lumen face of the cells forming the vessels. Costaining with CD34 (figure 1D, green) generated an orange colour where IR for both CD34 and GLUT-1 occurred. Supplementary figure 1D1–D3 shows the separated and overlaid CD34 and GLUT-1 images presented in figure 1D.

To identify EPCs within the IH lesions, IHC staining for VEGFR-2, CD34 and CD133 was undertaken, as this set of markers has been shown previously to identify EPCs within the circulation.^{7,8} All proliferating IH sections showed strong IR for VEGFR-2, CD34 and CD133. Figure 1E, F shows representative immunostaining for these markers. Figure 1E shows IR for CD34 (green) to identify the endothelial cells that also expressed the haematopoietic stem cell marker, CD133 (figure 1E, red). IR for CD133 is predominantly expressed on the CD34 cells, with a few cells in the interstitium also staining for CD133. Notably, the cells of the pericyte layer do not express CD133. Supplementary figure 1E1–E3 shows the CD133, CD34 and merged images in separate panels to show more clearly that CD133 and CD34 are expressed by the same cells that form the endothelium. Figure 1F shows IHC co-staining for VEGFR-2 (red) and CD34 (green). Expression of CD34 is restricted to the plasma membrane on the luminal side of cells lining the vessels, as also shown in figure 1C–E. Figure 1F shows that VEGFR-2 is expressed by the CD34 cells that line the microvessel lumen. Supplementary figure 1F1–F3 presents the CD34 and VEGFR-2 staining in separate panels and shows that VEGFR-2 is expressed in both the cytoplasm and nucleus of the CD34 cells. The white arrowheads in the merged images presented in figure 1F identify cells that strongly express VEGFR-2 and show that these cells are also CD34⁺. Collectively, the staining presented in figure 1E,F shows that the endothelium expresses markers consistent with an EPC phenotype.

As EPCs are derived from haemangioblasts,²³ we sought to investigate whether the endothelium was also immunoreactive for the primitive mesodermal marker brachyury,¹² and for ACE, also called CD143.¹⁴ IR for these markers, in conjunction with markers for EPC can be used to identify haemangioblasts. Figure 1E, F shows staining of representative IH sections for brachyury and ACE. All proliferating IH sections analysed showed strong IR for ACE and brachyury. Figure 1G shows staining for CD31 (green) and brachyury (red). IR for brachyury was restricted to the cells of the endothelium, which also stained for CD31. Supplementary figure 1G shows the separate staining for CD31 and brachyury and demonstrates that IR for brachyury is restricted to the CD31⁺ endothelial cells. Figure 1H shows staining for ACE (green) and SMA (red). As shown previously, SMA identifies pericytes and does not stain the endothelium (figure 1C). Figure 1H shows that IR for ACE is localised to the endothelium and is absent from the interstitial cells and pericyte layer. The staining presented in figure 1 G&H supports the notion that the expression of haemangioblast associated proteins in the endothelium of IH as being potentially haemogenic.

It has been previously shown that haemangioblasts generate haematopoietic cells through a haemogenic endothelium stage and that the transcription factor TAL-1 is essential for the establishment of a haemogenic endothelium.¹⁷ To confirm that the IR demonstrated in figure 1G, H identifies a haemogenic endothelium, staining for TAL-1 was undertaken and is presented in figure 1I. All proliferating IHs show strong IR for TAL-1 (red) on the CD34⁺ (green) cells that form the endothelium (figure 1I). Both nuclear and cytoplasmic staining of

TAL-1 was observed, with the cells forming the endothelium showing the greatest IR for TAL-1 (white arrowheads). To further support the presence of a primitive haemogenic endothelium, staining for GATA-2, a transcription factor expressed early in haematopoietic development, was undertaken. As for TAL-1, all proliferating IHs showed strong IR for GATA-2. Staining of a representative sample is presented in figure 1J. IR for GATA-2 (red) was seen in both the nucleus and cytoplasm of the CD34⁺ (green) cells forming the endothelium. Supplementary figure 1J1–J3 shows the separate staining for GATA-2 and CD34 that more clearly demonstrate the cytoplasmic and nuclear staining seen for GATA-2. Figure 1I,J shows that the transcription factors TAL-1 and GATA-2, which have been previously shown to identify primitive haematopoietic cells, are expressed by the CD34⁺ endothelium and are consistent with the data presented in figure 1G, H. Collectively, the data presented in both figure 1 and supplementary figure 1 show that the microvessels in proliferating IH express proteins associated with a haemogenic endothelium.

Once we had detected primitive haematopoietic markers in IH, we sought to determine whether the endothelium also expressed more mature haematopoietic markers. Figure 1K shows staining of a representative section for the mature haematopoietic cell marker, CD45.²⁴ Figure 1K shows a representative section stained for CD31 (green), and for CD45 (red). The section shows the characteristic strong IR for CD31, as expected. The white arrowheads indicate a selection of the CD45 cells, all of which are in the interstitial space between the CD31⁺ microvessels. IR for CD45, the mature myeloid cell marker, was restricted to interstitial space and was not detected on the endothelium of any of the proliferating IHs studied.

The specificity of the presented IR was confirmed by staining of placenta, which showed IR for CD34, CD133, ACE and GATA-2 (supplementary figure 2A–D) while there was no or minimal IR when staining of uterine fibroid tissues was performed as a negative control under identical conditions (supplementary figure 2E, F). Similarly, omission of the primary antibody when staining IH sections showed minimal IR (supplementary figure 2G, H).

DISCUSSION

Haemangioblast had remained elusive until 1998, when its presence as a common precursor for endothelial and haematopoietic cells was reported.²⁵ Since its discovery, proteins associated with haemangioblast development have been described²⁶ and subsequently used to identify cells with the same, or similar, phenotype based upon the expression of transcription factors and cell surface receptors. The nuclear transcription factor TAL-1 (also known as SCL) has been shown to be essential in specifying the formation of haemangioblasts from the primitive mesoderm,²⁷ and is also a key regulator of haematopoiesis.²⁸ Similarly, the zinc-finger transcription factor, GATA-2, has been shown to be a tissue-specific transcription factor whose activity is essential for cells with haematopoietic potential.²⁹ The nuclear and cytoplasmic localisation of this transcription factor in the cells of the endothelium that we report suggests that, although present, some of this transcription factor is not active and therefore localised outside the nucleus. The brachyury gene, also known as *T*, codes for a transcription factor that is expressed by cells derived from the primitive mesoderm,¹² the cytoplasmic localisation of this transcription factor has been previously reported in early development.³⁰ Haemangioblasts have previously been shown to express VEGFR-2, ACE, CD133, CD34 and CD31. We report that the

brachyury⁺ cells lining the immature capillaries in proliferating IH also demonstrate IR for TAL-1, VEGFR-2, CD133, CD34 and CD31, indicating that the cells lining the capillary lumen are derived from cells with haemangioblast capacity. The collective expression of the aforementioned transcription factors and cell surface markers is restricted to the cells lining the microvessel lumen, supporting the presence of a haemogenic endothelium as seen in blood islands of the developing human placenta.³¹ We have also recently shown that cells derived from proliferating IH have the ability to form blast colonies in vitro,¹⁰ which have been shown to be derived from primitive mesoderm,³² which in haemangioma has a neural crest stem cell phenotype.¹¹ Consistent with the primitive origin is the absence of staining for the panhaematopoietic marker CD45 by the endothelium,²⁴ although cells showing IR for CD45 are present in the interstitium between vessels.

Our results confirm, and add to, previous reports showing positive immunostaining in the immature capillaries of proliferating IH for markers associated with endothelial (VEGFR-2³³ TIE-2,³³ Ulex,⁴ von Willebrands factor,^{1 4} CD31,^{1 4 34 35} CD34^{2 34}) and haematopoietic (CD133³⁶ and CD34^{2 34}) lineages. Haemangioblasts have been identified and generated from embryonic stem cells,¹³ and have been shown to have the dual ability to form cells of both the endothelial and haematopoietic lineages. These data are consistent with the notion that primitive mesoderm gives rise to haemangioblasts that in turn generate the dense network of immature capillaries lined by plump immature endothelial cells within proliferating IH. To our knowledge, this is the first report demonstrating co-expression of brachyury, TAL-1, ACE and GATA-2 in the cells lining the immature capillaries of proliferating IH. The notion that haematopoietic cells are derived from an endothelial phenotype has formed the basis for the concept of the haemogenic endothelium,^{37 38} with the expression of TAL-1 being a crucial transcription factor for both the establishment and development of this haemogenic endothelium from haemangioblasts.¹⁷ It is interesting to speculate that the capillaries that predominate in proliferating IH are the structural haemogenic endothelium based on the collective expression of the primitive mesodermal and haematopoietic markers, used in this study, on the endothelium of these capillaries.

EPCs within proliferating IH⁸ have been suggested to be associated with increased levels of these cells in the peripheral circulation of affected patients.⁹ This has led to the suggestion that patients with IH have an inherent increased pool of EPCs that are then recruited into their IH, resulting in subsequent proliferation of the immature endothelial component of this tumour.⁸ This hypothesis implies that the establishment of IH lesions from circulating EPCs would require upregulation of primitive markers and dedifferentiation to a more primitive phenotype to form the haemogenic endothelium. The dogma that EPCs are primarily produced in the bone marrow, and recruited for tumour growth, has come under increasing scrutiny.³⁹ The expression of haematopoietic markers on the endothelium of proliferating IH shown in our study is more primitive than the markers expressed by circulating EPCs,^{8 9} supporting our hypothesis that endothelial progenitors originate from within the lesion. We infer that the more definitive downstream EPCs arise from the haemogenic endothelium and are released to the circulation. The expression of proteins associated with dual endothelial and haematopoietic lineages, and the observation of distinct mature endothelial cells¹ and myeloid cells⁵ in involuting IH, have led us to speculate that the dual endothelial and haematopoietic lineages seen during the development of IH may be attributed to a common haemangioblast precursor giving rise

Take-home message

The expression of primitive mesodermal, endothelial and haematopoietic markers by the cells forming the endothelium of infantile haemangioma suggests that the immature capillaries that predominate in proliferating lesions are a haemogenic endothelium phenotype, derived from haemangioblasts.

to a haemogenic endothelium intermediate. We further propose that the increased levels of circulating EPCs seen in patients with IH are derived from the haemogenic endothelium de novo and are released into the circulation.

The presence of the haemangioblast-derived haemogenic endothelium in IH suggests that this tumour may be best considered as a disorder of embryonic development, and that IH is a disorder of primitive mesodermal differentiation and proliferation. Our hypothesis is consistent with other studies reporting the presence of cells expressing proteins associated with embryonic haemangioblast progenitors in haemangioblastoma⁴⁰. Our recent observation that this haemogenic endothelium expresses a primitive mesoderm with a neural crest phenotype^{10 11} may provide a clue for the segmental distribution of a subgroup of IH including those that constitute PHACES (posterior fossa malformations—haemangiomas—arterial anomalies—cardiac defects—eye abnormalities—sternal cleft and supraumbilical raphe) syndrome.¹¹ Given that neural crest cells undergo extensive migration during embryonic development and that its derivatives are ubiquitous at birth, further study may provide insights into the localised form of IH, either single or multiple and/or extracutaneous involvement.

An understanding of the development of IH may not only provide insights into haemogenic endothelium and downstream human haematopoietic and vascular ontogeny but also provide a novel human model for studying primitive haematopoiesis and embryonic development in general.

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