Journal of Pathology
J Pathol 2003; **201**: 296–302.
Published online 22 July 2003 in Wiley InterScience (www.interscience.wiley.com). **DOI:** 10.1002/path.1443

Original Paper

Differential expression of CD146 in tissues and endothelial cells derived from infantile haemangioma and normal human skin

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Received: 11 March 2003
Revised: 14 April 2003
Accepted: 22 May 2003

Abstract

Haemangioma is the most common tumour of endothelial origin, occurring in 4–10% of Caucasian infants. It is characterized by rapid growth during the first year of postnatal life, followed by spontaneous regression from 1 to 7 years of age. The cell surface adhesion molecule CD146 has been identified as an endothelial cell marker. Despite advances in understanding the functional role of CD146 in normal endothelial cells and tumour progression, its expression and a possible role in an endothelial tumour have not been studied. As part of an investigation of endothelial cell alterations in infantile haemangioma, differential expression studies were performed with several known antigens and endothelial cell markers. Using immunohistochemical and flow cytometric analyses, cultured human dermal microvascular endothelial cells isolated from newborn foreskin (HDMEC) were compared with endothelial cells derived from haemangioma tissue (HemECs). In addition, immunohistochemistry was used to compare haemangioma tissues with normal human skin. Unexpectedly, cultured HemECs showed a significantly lower level of CD146 than HDMECs by both flow cytometric analysis and immunofluorescence staining. Using immunohistochemical studies, it was further demonstrated that endothelia in all haemangioma tissues, regardless of the tumour phase, showed negative immunoreactivity for CD146. In contrast, strong positive staining for CD146 was observed in the pericyte-like cells that surround the endothelial layers. These findings are believed to be relevant to the molecular basis of haemangioma. Furthermore, it is possible that antibodies against CD146 may be useful for separating haemangioma-derived endothelial cells from normal endothelial cells and pericytes. Copyright © 2003 John Wiley & Sons, Ltd.

Keywords: **benign tumour; haemangioma; endothelial cells; CD146; pericyte-like cells**

Introduction

CD146 is a transmembrane glycoprotein belonging to the immunoglobulin superfamily [1,2]. It is constitutively expressed in vascular endothelium, regardless of the vessel size and anatomical site. It is also present on several other cell types, including peripheral nerve cells, smooth muscle cells [3], intermediate trophoblasts [4], and mammary epithelial cells. CD146 expression has also been detected in some neoplasms including melanoma, angiosarcoma, Kaposi sarcoma, and leiomyosarcoma [5]. Although the exact role of CD146 in endothelial function is not known, there is evidence to support the notion that it acts as a cell–cell adhesion molecule [6]. The possible function of CD146 in tumours is conflicting. CD146 expression has been shown to correlate with the progression and metastatic potential of melanoma [1,7]. It has been suggested that CD146-negative cutaneous melanoma cells have increased tumourigenicity and metastatic potential in nude mice after transfection with CD146 cDNA [8]. However, transfection of the CD146 cDNA into CD146-negative breast carcinoma cells produced smaller tumours in SCID mice, which were more cohesive and less infiltrative [9].

CD146 was originally found expressed in human melanomas and only later was it defined as an endothelial cell marker. In this study, we demonstrate that CD146 is not expressed in the endothelium of haemangioma tissue. In contrast, pericyte-like cells surrounding the endothelium in haemangioma show strong immunoreactivity. Further studies of CD146 expression in haemangioma may provide insight into the biological significance of this molecule in the pathogenesis, progression and regression of this common tumour.

Materials and methods

All of the studies involving human materials and subjects were approved by the HMS/HSDM Committee

on Human Studies (Assurance No M1240-01) — Human Studies Docket No X101896-1.

Cell culture

Endothelial cells were isolated from proliferating phase infantile haemangiomas (HemECs) and normal human newborn foreskin (HDMECs) as previously described [10]. The cells were cultured in gelatin-coated tissue culture dishes and maintained in EBM (Clonetics, San Diego, CA, USA) containing 10% FBS (Hyclone Laboratories), 100 U/ml penicillin, 100 μg/ml streptomycin (Irvine Scientific), and 2 ng/ml bFGF (R&D System, Inc). For immunostaining, both HemECs and HDMECs were grown on gelatin-coated glass coverslips (13 mm in diameter) and fixed with acetone–methanol at a 1 : 1 ratio for 15 min at −20 °C and processed for indirect immunofluorescence microscopy, as described below.

Immunohistochemistry

All the secondary antibodies were purchased from Vector Laboratories, unless otherwise indicated. Cryostat sections from haemangiomatous tissue and normal human foreskin were fixed in acetone–methanol at a 1 : 1 ratio for 15 min at −20 °C. Sections were permeabilized in 0.25% Triton–PBS for 4 min, subsequently treated with 1% H_2O_2 for 10 min, and blocked in 1% BSA (Sigma) for 1 h at room temperature. Primary antibodies, anti-CD146 (PIH12, 1 : 200; Chemicon International), anti-Glut-1 (1 : 800; DAKO), and anti-CD31 (1 : 1000; Santa Cruz) diluted in PBS–1% BSA, were applied to the sections and the sections were incubated for 1 h at room temperature. Matching isotype IgG was used as a negative control. Sections were washed in PBS three times, 5 min each, and incubated with biotinylated secondary antibodies for 30 min. After intensive washing in PBS, the sections were incubated with ABC solution (Vector Laboratories) for 30 min and reacted with DAB substrate. For double fluorescence labelling, tissue sections were incubated with a mixture of the two antibodies, goat anti-CD31 (1 : 1000; Santa Cruz) or rhodamine-coupled *Ulex europaeus agglutinin* 1 lectin (UEA1, 1 : 200) and mouse anti-CD146 (1 : 200) or α-smooth muscle actin (α-SMA, 1 : 400; Sigma), for 1 h at room temperature, followed by three PBS–0.1% Triton X-100 washes. Texas Red-conjugated anti-goat for CD31 (1 : 200) and FITC-coupled anti-mouse secondary antibody for α-SMA or CD146 (1 : 200) was applied to the sections, respectively, and incubated for 30 min at room temperature. Sections were mounted with anti-fading reagent (Vector Laboratories). A Nikon E800 microscope was used for fluorescence and image analysis.

Flow cytometric analysis

HDMECs and HemECs were trypsinized and resuspended in PBS–0.1% BSA. Cells were incubated with anti-CD146 (5 μg/ml) or mouse control IgG (5 μg/ml) (Santa Cruz) for 40 min at 4 °C. Alternatively, 0.2 μl/ml FITC-UEA1 (Vector Laboratories) was added. After three washes in PBS–BSA, the cells incubated with anti-CD146 and control mouse IgG were incubated with FITC-conjugated anti-mouse secondary antibody (1 : 200) for 30 min at 4 °C. Following additional washing, cells were fixed with 1% paraformaldehyde in PBS for 1 h at 4 °C and finally resuspended in 0.5 ml of PBS–BSA–0.02% sodium azide at a density of 1×10^6 cells/ml. For dual-colour flow cytometric analysis of co-cultured HemECs and HDMECs, HemECs were labelled with 2 μM PKH26 in diluent C, a red fluorescent cell linker (Sigma, MINI-26). The same amount of diluent C without dye was added to HDMECs as a control. Labelled HemECs and non-labelled HDMECs were resuspended in the complete culture medium at a concentration of 1×10^6 cells/ml, respectively, and a mixture of the two cell types was made by mixing equal volumes of HemECs and HDMECs. The cells were further cultured for 24 or 48 h before analysis for CD146 or UEA1 staining as described above. Samples were analysed by FACScan (Becton Dickinson).

Results

CD146 expression in cultured HemECs and HDMECs

Flow cytometric analysis showed that two HemEC lines (HemEC1 and HemEC26) had significantly lower CD146 expression levels than HDMECs (Figure 1A). Five HDMEC lines examined demonstrated consistently high levels of CD146 expression (Figure 1B). HemECs or HDMECs exhibited variable UEA1 levels (Figures 1C and 1D). Indirect immunofluorescence staining for CD146 in cultured ECs showed the same findings (data not shown). CD146 is mostly present on the cell surface, but is also seen intracellularly. FACS analyses for merosin, FcγRII, and GLUT1 failed to show any significant differences between HemECs and HDMECs (data not shown).

CD146 expression in co-cultured HemECs and HDMECs

It has been proposed that CD146 expression in cultured endothelial cells may correlate with cell density, with increased levels of CD146 being seen at increased cell densities [6]. To verify that the difference in CD146 expression between HDMECs and HemECs is intrinsic and not caused by differences in cell density, we performed co-culture experiments and dual-colour flow cytometric analysis. HemECs were pre-labelled with PKH26, a red fluorescence cell tracer, and an equal number of HemECs and HDMECs were mixed and co-cultured for 24–48 h. In Figure 2, the panels on the left (A–C) show that in the co-culture of two cell types, there is a clear shift of CD146 intensity in

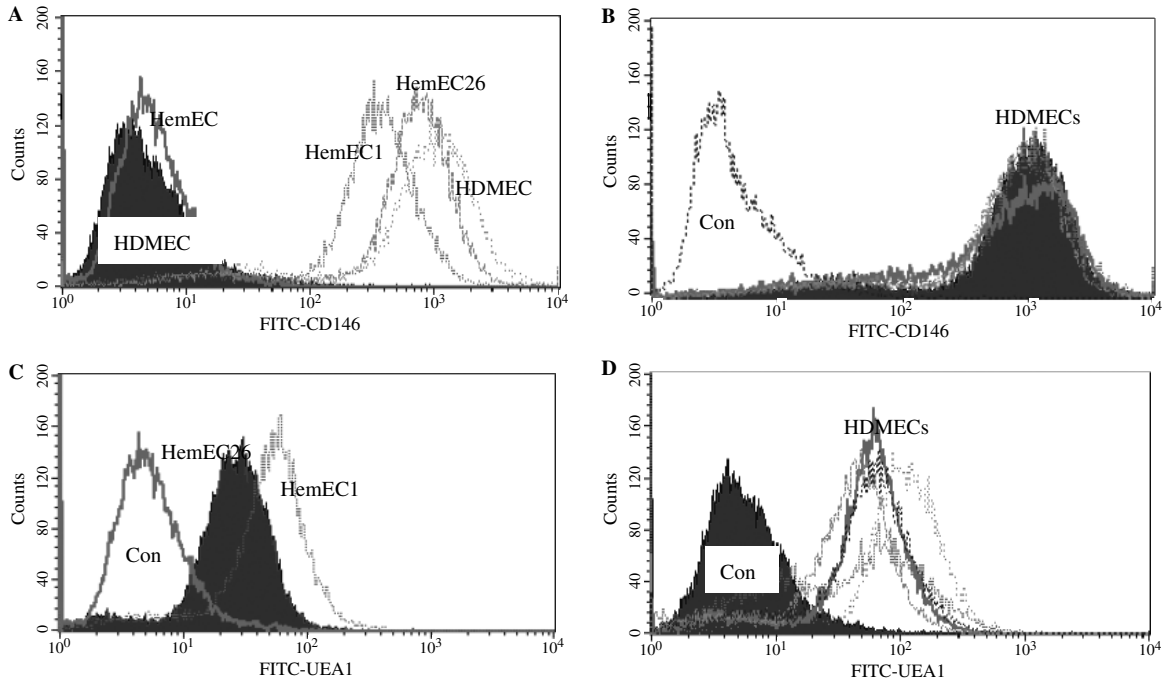

Figure 1. Flow cytometric comparison of CD146 (A, B) and UEA1 (C, D) expression in cultured HDMECs and HemECs. (A) Two HemEC lines (HemEC1 and HemEC26) show a lower level of CD146 than HDMECs. The graphs in green and purple represent cells that were stained with control IgG. (B) Five HDMEC lines revealed a consistent expression level of CD146. Con represents cells that were stained with control IgG. (C, D) Both HDMECs and HemECs show variable expression of UEA1

the mixed cell population determined by FITC intensity after 24 h (Figure 2B). Population Y represents the HemEC line; population X represents HDMECs. The same shift pattern was observed after 48 h of co-culture (data not shown). A shift along the x-axis was observed in both cell populations when stained with UEA1 (Figure 2C), indicating that the two cell populations express similar levels of UEA1, as determined by FITC intensity. The panels on the right (D–F) represent single cell types and show the same findings as those described above.

Immunohistochemical staining for CD146, CD31, and GLUT1 in tissues

We next examined haemangioma and normal human foreskin tissues. Cryostat sections of haemangioma tissue from different phases and normal human skin were stained with CD146, CD31, GLUT1, merosin, and FcγRII antibodies. Antibodies against CD146 (Figures 3A and 3B), CD31 (Figures 3C and 3D), and GLUT1 (Figures 3E and 3F) showed positive staining. Interestingly, the staining pattern for CD146 and CD31 differed, as recognized at higher magnification (in the right panel, 40×). CD146 expression was absent in the luminal endothelium of vessels in haemangioma tissue (Figure 3B, arrow-head), whereas CD31 staining was clearly positive in ECs of the same tissue (Figure 3D, arrow). Consistent with previous reports [11,12], merosin, FcγRII (data not shown), and GLUT staining was intense in haemangioma tissue (Figures 3E and 3F).

Double immunofluorescence labelling of CD146 with other endothelial and smooth muscle cell markers

To confirm that the endothelium in haemangioma tissue does not express CD146, we performed double labelling with anti-CD146 and the endothelial-specific markers, either CD31 or UEA1. We did not observe an overlap between either UEA1 or CD31 and CD146 staining (Figures 4A and 4C). Clearly, rhodamine-conjugated UEA1 and Texas Red-conjugated anti-CD31 stained the luminal endothelium of the vascular channels, whereas FITC-labelled anti-CD146 stained the surrounding pericyte-like cells. Indeed, the fluorescence signal seen when double labelling was performed with anti-CD146 (green) and UEA1 (red) was identical to the staining pattern observed with anti-α-SMA (green) and anti-CD31 (red) (Figure 4B) to highlight perivascular cells (green) and the endothelium (red). However, an overlap of CD31 or UEA1 and CD146 staining was seen in normal human skin capillaries (Figures 4C and 4D, arrow-heads). The staining pattern of CD146 was the same in proliferating phase (Figure 5A) and involuting phase specimens (Figure 5B).

Discussion

CD146 is a cell surface adhesion molecule that was originally cloned from human melanoma [13–15]. Its expression is associated with malignant behaviour and progression of this tumour. In contrast, CD146 was

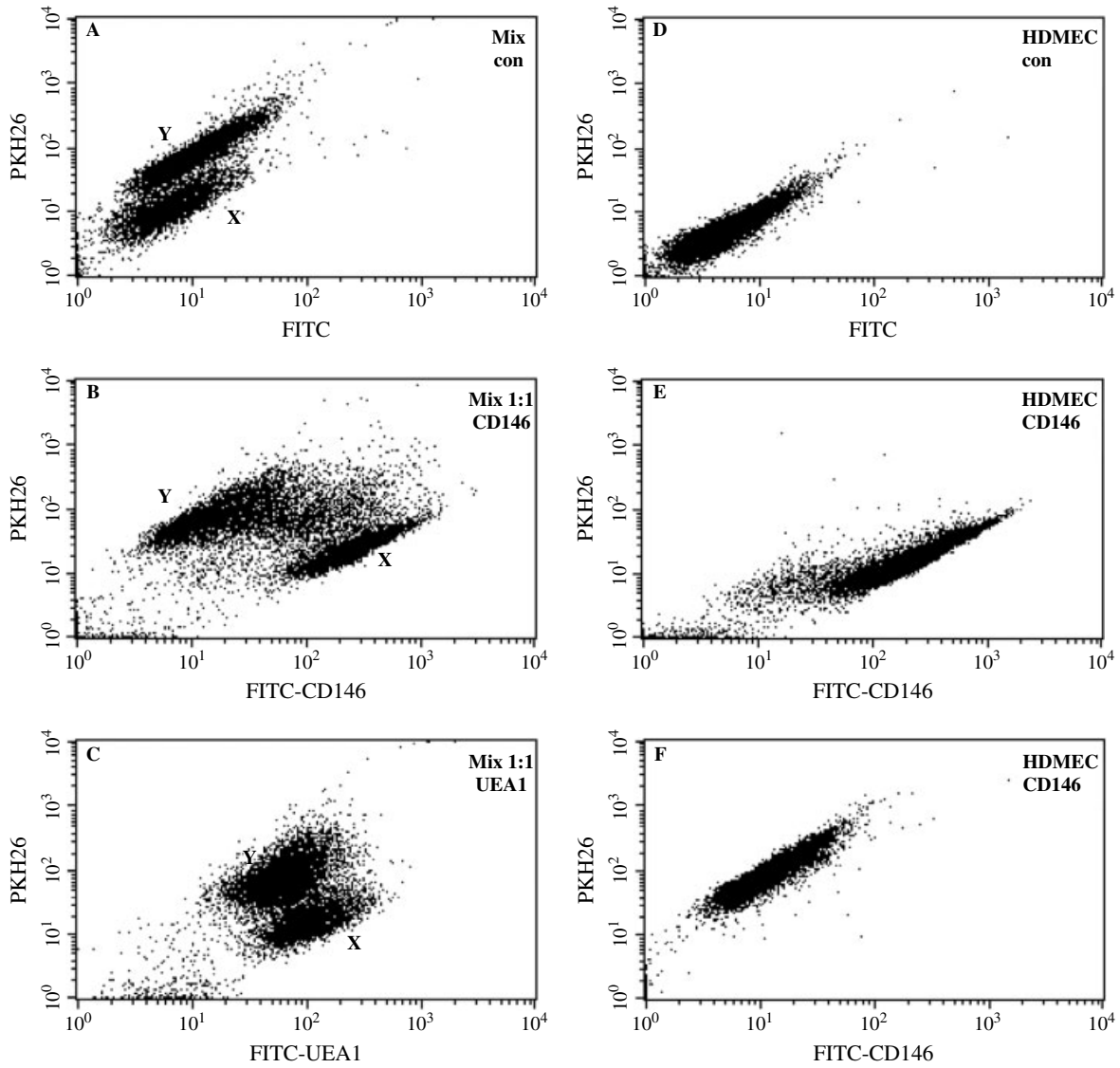

Figure 2. Dual-colour flow cytometry for CD146 and UEA1 expression. HemECs were labelled with the red fluorescence PHK26 cell linker (*y*-axis). Cells were stained with anti-CD146 antibody or UEA1 (FITC-conjugated, *x*-axis). (A–C) A 1 : 1 mixed cell population of HemECs and HDMECs was co-cultured for 24 h, followed by analyses for CD146 and UEA1 expression. Population Y represents HemEC21. Population X represents HDMECs. A clear shift is seen in CD146 staining (B). (D–F) Single cell type stained for CD146. (D) Cells were stained with control IgG. (E) HDMECs were stained with anti-CD146 antibody. (F) CD146 expression in PKH26 labelled HemEC21

expressed in only 27% of breast carcinomas, while it is normally expressed in breast glandular epithelium [9]. More recently, CD146 was found to be present in the endothelium of human vasculature and recognized as an endothelial cell marker [16,17]. Although its exact role is not clear, CD146 is localized at the endothelial junctions, where it may be involved in the regulation of paracellular permeability and signal transduction of endothelial cells [6,18,19]. It has also been reported that CD146 expression in culture correlates with endothelial cell density [6]. In this study, we have demonstrated that endothelia of the vascular channels in haemangioma tissue are negative for anti-CD146 staining. In contrast, pericyte-like cells surrounding ECs show strong immunoreactivity. Three HemEC lines had significantly lower levels of CD146 expression than five HDMEC lines. This difference was further confirmed in co-culture experiments by dual-colour FACS analysis, suggesting that the difference in CD146 expression is intrinsic to haemangioma endothelial cells and is not caused by differences in cell density.

The molecular pathogenesis and mechanisms responsible for the evolution of infantile haemangioma are of considerable biological interest. Recent studies have revealed that haemangiomas consist of clones of ECs that are functionally different from normal microvascular ECs *in vitro*. HemECs proliferate and migrate faster than HDMECs in culture [20]. Furthermore, endothelial cells in haemangioma tissue exhibit altered expression of several angiogenesis-related molecules, such as E-selectin, Tie2, VEGF, bFGF, and IGF2 [21–23]. All these changes, as well as down-regulation of CD146 expression, may be

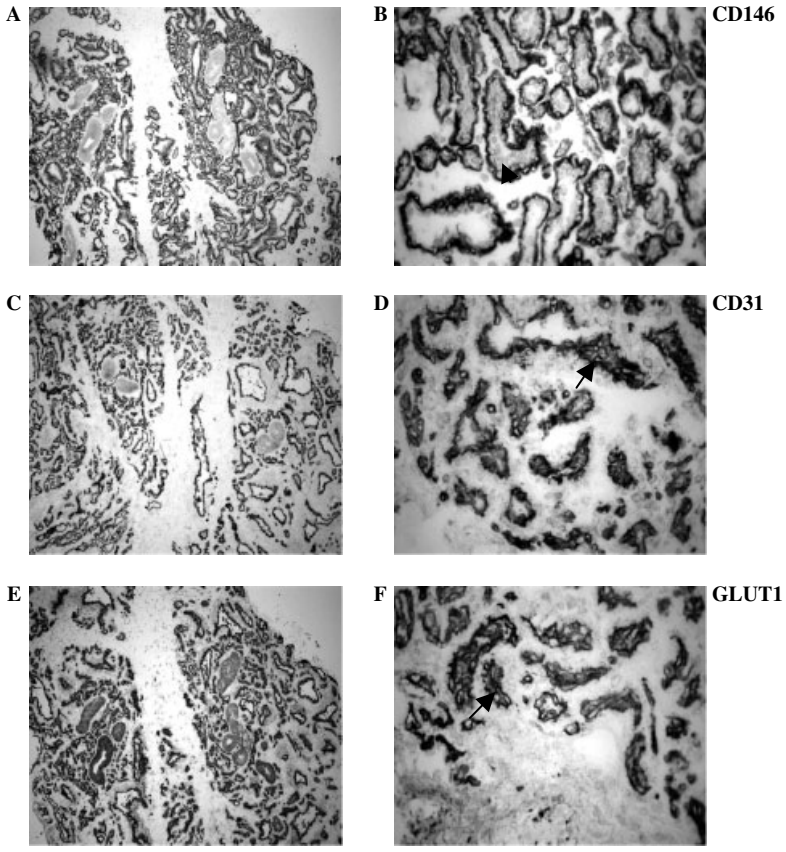

Figure 3. Immunohistochemical staining of haemangioma tissue. The panels on the left are low magnification (10×). The panels on the right are 20×. (A, B) Immunostaining for CD146. Note that the inner layer of endothelial cells is immunonegative, while surrounding pericyte-like cells are immunoreactive. (C, D) Tissues were stained with anti-CD31 antibody. Panels E and F reveal a similar positive staining pattern for GLUT1

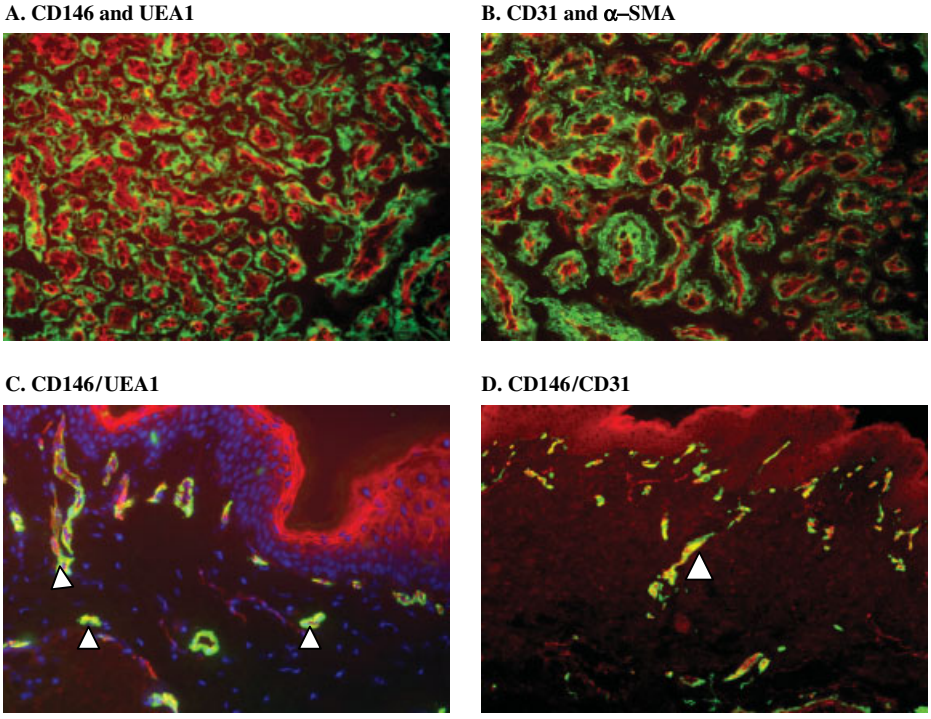

Figure 4. Double immunofluorescence staining of haemangioma (A, B) and normal human foreskin (C, D) tissue for endothelial and smooth muscle cell markers. (A) Endothelium was stained with UEA1 labelled with red fluorescence, while anti-CD146 conjugated with FITC was present in the outer layer of the vessels. (B) Pericyte-like cells were stained with FITC-labelled anti-α-SMA; endothelium was stained with anti-CD31 conjugated with red fluorescence. (C, D) An overlap between CD31 (red) or UEA1 (red) and CD146 (green) was seen in the dermal capillaries

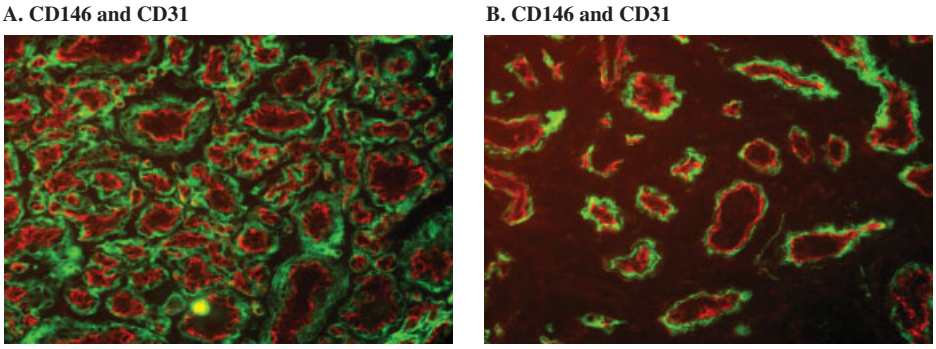

Figure 5. Double fluorescence labelling for CD31 and CD146 expression in different phases of haemangioma. (A) Proliferative phase, Texas red-labelled anti-CD31 and FITC-conjugated CD146 do not overlap. (B–D) Involuting phase; the vessels show the same staining pattern

a consequence of a genetic alteration in haemangioma endothelial cells. Thus, CD146 could be another molecule that can be used to distinguish HemECs from normal ECs. However, it differs from previously described markers such as merosin, GLUT1, FcγRII, and Lewis Y antigen [11,12], in that the low-level expression of CD146 in haemangioma tissue is maintained after HemECs are isolated and cultured *in vitro*. In contrast, merosin, GLUT1, and FcγRII are expressed at high levels in fresh or fixed haemangioma tissue, but their levels of expression in HemECs become indistinguishable from that of normal ECs in culture (data not shown).

Given the potential role of CD146 as an adhesion molecule at intercellular junctions of normal ECs and the plump, abnormal morphology of endothelial cells in haemangioma, it is possible that the low-level expression of CD146 contributes to the haemangioma vascular phenotype. It is conceivable that adhesion molecules, such as CD146, not only glue endothelial cells together, but are also involved in signal transduction and regulation of cellular functions. Most transmembrane adhesion molecules are linked to a network of cytoplasmic/cytoskeletal proteins. It has been reported that CD146 initiates an outside–in signalling pathway involving the protein tyrosine kinases FYN and FAK, as well as paxillin, that leads to the association of CD146 with the cytoskeletal network in human umbilical vein endothelial cells [6]. It would therefore be of interest to determine whether up-regulation of CD146 expression in HemECs normalizes, partially or completely, the functional abnormalities in cellular proliferation and migration, which we have reported for HemECs [20].

Acknowledgements

Partial support was provided by NIH grant AR36820 (to BRO) and the John B Mulliken Foundation.

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