Leukocyte Antigen CD34 is Expressed by a Subset of Cultured Endothelial Cells and on Endothelial Abluminal Microprocesses in the Tumor Stroma


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It has been reported that the human haemopoietic progenitor cell antigen CD34 is also expressed by vascular structures. To investigate its precise vascular localization, we have studied the cellular and subcellular distribution of CD34 in normal tissues and pathologic tissues with neovascularization. In normal resting tissues, anti-CD34 antibodies, ICH3 and QBEND-10 predominantly stain the luminal endothelial membrane, whereas the abluminal membrane is negative or weakly positive. In contrast, a striking staining of endothelial abluminal microprocesses (EAM) was found in the tumor stroma. These structures, measuring up to 20 μm in length, could be observed in thick vibratome sections both at the tips of vascular sprouts and, also frequently, on fully formed microvessels. The number of vascular sprouts and EAM varied widely between different tumors. CD34-stained EAM were sparsely present in fetal tissue of 10 weeks gestation, but they could not be demonstrated in granulation tissue of wound healing. By immunoelectron microscopy, the EAM were continuous with the cytoplasm of endothelial cells showing an immature phenotype as seen in regeneration. In cultured human umbilical vein endothelium, CD34 was preferentially found on a small subset of cells with the morphologic appearance of migrating cells. These findings suggest that CD34 is an endothelial marker for EAM present during angiogenesis.

Additional key words: Endothelium, Wound healing, Angiogenesis.

The human haemopoietic progenitor cell antigen CD34 is known to be synthesized and expressed by certain cells of hemopoietic lineage (4, 5, 17, 35, 40). Furthermore, anti-CD34 monoclonal antibodies (MoAbs) have been reported to stain blood vessels in tissue sections (4, 40). However, it has remained unclear whether this vascular staining was due to cross-reacting epitopes or to production and expression of the CD34 molecule proper by endothelial cells.

In order to elucidate this phenomenon we have investigated the subcellular distribution of this antigen in cultured human endothelial cells and in the vasculature of normal human tissues and tumors. The investigations reveal staining in tissues for CD34 on the luminal endothelial membrane and of abluminal endothelial microprocesses (EAM) associated with vascular pruning during angiogenesis.

Angiogenesis is characterized by a series of events, i.e., migration of endothelial cells, followed by sprout formation, endothelial proliferation, fusion of sprouts, and eventually maturation of the newly formed vessel by formation of basement membrane and the appearance of pericytes (2, 6, 7, 13–16, 18, 32). In embryogenesis and wound healing this process is well-regulated and ceases when tissue repair or organ development is completed (16). In contrast, angiogenesis in the tumor stroma seems to be continuously induced by the growing tumor (11, 13, 28, 39).

In early morphologic studies on wound healing and embryogenesis, it was observed that endothelial cells during the initial migration step of angiogenesis project abluminal extensions to the surrounding interstitium (7, 32). We now demonstrate by CD34 staining the presence of equivalent processes in the human tumor vasculature.
which we would like to call EAM. In addition we report on the preferential expression of CD34 on a small subset of cultured human endothelial cells in vitro.

EXPERIMENTAL DESIGN

The distribution of the CD34 antigen in the microvascularity of tumors was compared with that in granulation tissue of wound healing, normal human fetal tissues, and normal human adult tissues by immunohistochemistry using anti-CD34 MoAbs ICH3 and QBEND-10 on frozen tissue and thick vibratome sections.

The subcellular distribution of CD34 was studied by an immunohistochemical analysis on the electron microscopic (EM) level.

In order to find evidence for production of the CD34 molecule by endothelial cells, immunohistochemistry on the light microscopic and EM level was performed on cultured human umbilical vein endothelium.

The influence on CD34 expression of agents known to affect endothelium during angiogenesis, e.g., tumor necrosis factor, the phorbol ester, phorbolmyristate acetate and co-culture with tumor cells on cultured endothelium was assessed.

RESULTS AND DISCUSSION

THE CD34 ANTIGEN IS EXPRESSED BY A SUBSET OF CULTURED ENDOTHELIAL CELLS

Our results provide the following evidence that CD34 is synthesized by endothelium: (a) Anti-CD34 MoAbs, ICH3 and QBEND-10 markedly stain a subset (5 to 20%) of cells in monolayers and suspensions of human umbilical vein endothelial cells (HUVEC) (Figs. 1 and 2). In monolayers, the CD34-staining cells seem to lay on top of the endothelial monolayer and they show bidirectional extensions. Staining was performed with cells at serial passage number 5 to avoid contamination with nonendothelial cells. Double-labeling with a polyclonal antibody directed against von Willebrand Factor confirmed the endothelial nature of the CD34-stained cells (data not shown). Staining with irrelevant antibodies (e.g., anti-CD34 MoAb WT32, anti-HLA class II MoAb Q2/70 and MoAb RC38) was negative in all cells. Cultured human brain pericytes, smooth muscle cells and melanoma cells failed to stain with anti-CD34 MoAbs. (b) The anti-CD34 MoAbs used in this study recognize distinct epitopes on the CD34 molecule (40). Still, we observed identical staining patterns of the two MoAbs both in cultured endothelial cells and in tissues.

Other authors have shown that anti-CD34 MoAbs, My10, 12.8, and B1.3C5 (recognizing different epitopes on the CD34 antigen) react with blood vessels in tissue sections (4, 40) as do all (of 6) recently identified CD34 antibodies (L. Fina, unpublished data). Moreover, CD34 cDNA was recently cloned and corresponding mRNA could be demonstrated by northern blotting in our endothelial cells (L. Fina, unpublished data). All these data strongly suggest that endothelial cells synthesize a molecule similar to the haemopoietic progenitor CD34 antigen. Incubation of the endothelial cultures with tumor necrosis factor or cultured colon carcinoma cells, stimuli capable of influencing the angiogenic process in vivo (23), or addition to the culture medium of the phorbol ester, phorbolmyristate acetate, an agent modulating cellular differentiation, did not affect the percentage of CD34-staining endothelial cells (data not shown).

NORMAL TISSUES: POLARIZED EXPRESSION OF CD34 ON ENDOTHELIOUM

Light Microscopy. Overall staining of the vasculature was observed in both frozen and thick vibratome sections of normal tissues stained with anti-CD34 MoAbs, ICH3 and QBEND-10 (shown in Fig. 3). By light microscopy,
no clear distinction between luminal and abluminal endothelial staining could be made. A detailed tissue distribution of anti-CD34 MoAb, QBEND-10 or a series of anti-CD34 MoAbs will be presented in separate papers (N. J. Bradley and L. Fina, unpublished data).

Electron Microscopy. At the subcellular level, a marked, evenly distributed staining of the luminal endothelial membrane was observed in blood vessels of normal tissues. In contrast, the abluminal membrane of the endothelial cells was only weakly stained, indicating a polar expression of CD34 in endothelium in vivo.

TUMOR AND FETAL VASCULATURE: EXPRESSION OF CD34 ON EAM

Light Microscopy. In frozen and thick vibratome sections of tumor tissues, anti-CD34 MoAbs, ICH3 and QBEND-10 markedly stained vascular structures, as is shown for MoAb ICH3 in Figures 4, 5, and 6. No reaction with tumor cells was seen. However, in addition to the overall vascular staining which was also observed in normal tissues, in the vibratome sections, striking CD34-staining of vascular abluminal microprocesses (Figs. 5 and 6) was observed. These structures were seen extending up to 20 \( \mu \)m into the surrounding tissue both from apparently blind-ending vascular sprouts (Fig. 6) as from other sites along fully formed vessels (Fig. 5). Often, microprocesses were connected with one site. In adjacent vibratome sections from the same lesions stained with MoAbs, PAL-E or EN-4, no abluminal microprocesses were observed. Similar microprocesses could be detected in vibratome sections of fetal tissues of 10 weeks gestation, being locally present in the microvasculature of the paravertebral and the cutaneous plexus (Fig. 7). EAM was not evident in fetal tissue of 18 weeks gestation or in granulation tissue of wound healing.

Electron Microscopy. A marked staining with anti-CD34 MoAbs, ICH3 and QBEND-10 of both the luminal endothelial membrane and of EAM was observed in tumor tissues (Figs. 8 and 9). The extensions were continuous with the endothelial cell membrane (Figs. 8 and 9) and occasionally branched. They extended mostly into electron-lucent areas of the interstitium (Figs. 8 and 9) and lacked basement membrane as shown in control sections stained for collagen type IV.

Comments

EAM have been observed by other authors in studies of wound healing and embryogenesis. They were first described by Klosovskii (19) and Schoefl (32). Klosovskii (19) showed by light microscopy that capillary sprouts have the aspect of polyps with tendril-like processes at their tips. Schoefl (32) demonstrated long cytoplasmic
The distribution pattern contrasts with our observation of sparsely distributed EAM in thick vibratome sections of fetal tissues of 10 weeks gestation and with observations by other authors of EAM solely at the tips of vascular sprouts in embryogenesis (20) and wound healing (32). It is also interesting that the prevalence of vascular sprouts and EAM varied widely in the various tumors studied. In one poorly differentiated adenocarcinoma of the colon and two neuroblastomas, EAM were numerous and could be observed on most vessels (Fig. 5). In other moderately and highly differentiated adenocarcinomas, sprouts and EAM were confined to just a few areas. These patterns did not differ in central as compared with peripheral parts of the tumors studied and are therefore a consistent property of a particular tumor. As the degree of angiogenesis may vary among the various tumors (13, 21, 29, 39), it may be interesting to study the frequency of EAM in relation to this process. Since the distribution of EAM in human tissues can be studied with anti-CD34 MoAbs, this method provides a new way of studying angiogenesis in situ. It is conceivable that this approach, lectins could also be useful (41).

We found that vascular structures and sprouts with endothelial abluminal processes in tumor stroma were often associated with pericytes. However, neither pericytes nor fibroblasts stained with anti-CD34 MoAbs. The close association of pericytes with sprouting endothelial cells is unexpected, as this does not fit in the classical model of angiogenesis (13, 16, 25), by which pericytes are absent from the tips of sprouts and present only in the stage of final vascular differentiation (8, 25, 34).

The structure and function of CD34 are unknown. Its expression in both human hematopoietic progenitor cells and endothelium is interesting in the light of the proposed early embryonic common origin of these cells (26). Previously, an antigen (MB1) was described in the quail, that is also shared by endothelial and hematopoietic cells (26). In conclusion, our results suggest that the CD34 antigen is a marker for EAM present during angiogenesis. Demonstration of EAM and their distribution in thick vibratome sections by staining with anti-CD34 monoclonal antibodies could provide a key method for studying angiogenesis in situ.

METHODS

Tissue Samples

Samples from normal and pathologic human tissues were obtained from fresh surgical specimens and autopsies obtained within 6 hours after death. The normal tissues and pathologic tissues are listed in Table 1. All tissues were snap-frozen and stored at −70°C.

For preparation of thick vibratome sections and immunoelectron microscopy, samples from eight colon carcinomas, two neuroblastomas, a mixed tumor of the parotid gland, a full-term placenta obtained at caesarean section, two cases of granulation tissue of wound healing, normal fetal tissues of 10 and 18 weeks gestation, (including striated muscle, vertebral column, limb, liver), normal adult kidney, bladder and prostate, were used within 30 minutes of surgical removal.
CULTURED CELLS

The microvascular endothelial cells were isolated from human foreskin as described by Davison, Bensch, Karasek (9) and cultured in Dulbecco's minimal essential medium supplemented with 20% normal human serum. HUVEC was isolated from umbilical cords and cultured as described by van Hinsbergh et al. (36). For experiments, fifth passage cells were used. Human brain pericytes were cultured from cerebral cortex obtained from autopsy patients in a modification of the method described by Vinters et al. (37) and Schlingemann et al. (32a). Briefly, microvessels were isolated from cerebral cortex by mechanical disruption (Dounce tissue grinder) followed by differential centrifugation and adherence to plastic surfaces. For this latter purpose, untreated Petri dishes were used instead of a column filled with beads. The capillary fragments were subsequently digested with collagenase (0.1% 10 to 20 minutes at 37°C) and seeded in culture flasks. Endothelial cell outgrowth was followed by outgrowth of pericytes, which could be freed of contaminating elements by limiting dilution. These cells were characterized as pericytes on the basis of their morphology and positive staining with MoAb 3G5 (a specific marker for pericytes, kindly provided by Dr. C. Nayak) (24). Cultured human smooth muscle cells, the human colon carcinoma cell line Colo 320 and the melanoma cell line M14 were kept at standard culture conditions. For studies of modulation of CD34 expression on HUVEC, third or fourth passage cells were seeded on plastic microscope coverslips coated with fibronectin kindly provided by Dr. J. van Mourik, Central Laboratory of the Red Cross Blood Transfusion Service, Amsterdam, The Netherlands. After 24 hours, the cells were incubated for another 24 hours with either 5 and 20 ng/ml of phorbolmyristate acetate (Sigma), 5, 20, and 100 nM recombinant tumor necrosis factor or were co-cultured with 10^4 Colo 320 colon carcinoma cells. The effect of the different incubations was evaluated by determining the percentage of cells staining with anti-CD34 MoAb QBEND-10.
IMMUNOHISTOCHEMISTRY

Air-dried 4-µm frozen tissue sections, culture cover slips, and cytospins of cultured cells were fixed with cold acetone for 10 minutes and stained by a sensitive indirect immunoperoxidase procedure (22, 33), using antibodies at appropriate dilutions in phosphate-buffered saline containing 1% bovine serum albumin. 3,3'-Diaminobenzidine tetrahydrochloride, 0.075% in Tris-buffered saline (TBS), pH 6.0, containing 0.001 M imidazole and 0.01% H₂O₂ was used as substrate (32a). In control sections, incubation with first antibody was omitted or replaced by an irrelevant monoclonal antibody (anti-CD3 MoAb WT32 anti-HLA Class II MoAb Q2/70 or MoAb RC 38 recognizing renal cell carcinomas). Sections were counterstained with hematoxylin for visualization of the nuclei.

IMMUNOELECTRON MICROSCOPY OF TISSUE SAMPLES

Small tissue specimens were fixed at room temperature for 4 hours in freshly prepared 4% paraformaldehyde in Sorensen phosphate buffer, pH 7.4, and washed overnight in TBS, pH 7.4. Vibratome sections (50-µm) were cut, washed in TBS, and incubated at 4°C overnight with MoAb ICH3 or MoAb QBEND-10 at appropriate dilutions in TBS containing 0.5% bovine serum albumin and 0.005% saponin. Incubations and washing steps were performed using a rotary shaker. After four 30-minute washings with TBS, the sections were incubated with a mixture of horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG and HRP-conjugated goat anti-mouse IgG for 2 hours at room temperature. Control sections were incubated with second antibodies alone. Serial sections were stained with MoAbs PAL-E and EN4, and with MoAb 1042, to enable exact localization of the basement membrane. After extensive washings in TBS, the sections were preincubated for 10 minutes in 0.75 mg/ml of 3,3'-diaminobenzidine tetrahydrochloride in TBS, pH 5.7, containing 0.001 M imidazole. Sections were then developed for 10 minutes in the same solution containing in addition, 0.01% H₂O₂; and the reaction was terminated by rinsing with TBS. Subsequently, the sections were postfixed for 40 minutes in 1% osmium tetroxide in phosphate buffer, then dehydrated, embedded in Epon 812 and processed routinely. Sections (1-µm) were cut for light microscopy. Ultrathin sections were cut and without further staining, examined and photographed on a Philips 300 electron microscope at 40 kv.

IMMUNOELECTRON MICROSCOPY OF CELLS

Cell suspensions of HUVEC were prepared by treating fifth passage endothelial monolayers with EDTA, 0.01% for 30 minutes at 37°C. Subsequently the cells were fixed for 10 minutes with 2% paraformaldehyde in Sorensen phosphate buffer, pH 7.4, and washed in TBS. After staining of the suspended cells with MoAb QBEND-10 or irrelevant antibodies and 3,3'-diaminobenzidine tetrahydrochloride as described in a preceding paragraph, the cells were spun down and further processed for electron microscopy as a pellet in a fashion similar to that of the stained vibratome sections.

MONOCLONAL ANTIBODIES AND CONVENTIONAL ANTISERA

The MoAbs ICH3 and QBEND-10 to distinct determinants of the CD34 molecule, the MoAbs PAL-E and EN4 to endothelial differentiation markers, MoAb 1042, recognizing collagen type IV, (kindly provided by Dr. F. T. Bosman, Department of Pathology, State University Maastricht, The Netherlands), anti-CD3 MoAb WT32, MoAb 3G5, MoAb Q2/70 and MoAb RC 38 were developed and characterized as described elsewhere (24, 30, 31). A polyclonal antibody recognizing von Willebrand Factor (factor VIII-related antigen), HRP-conjugated goat anti-mouse IgG antibodies and HRP-conjugated rabbit anti-mouse IgG.

**TABLE 1. TISSUES AND CULTURED CELLS USED IN THIS STUDY**

<table>
<thead>
<tr>
<th>Normal tissues</th>
<th>Pathologic tissues</th>
<th>Cultured cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney</td>
<td>Tumors</td>
<td>Foreskin microvascular EC*</td>
</tr>
<tr>
<td>Striated muscle</td>
<td>Neuroblastoma (N = 2)</td>
<td>Umbilical vein EC</td>
</tr>
<tr>
<td>Thyroid</td>
<td>Lung carc</td>
<td>Fibroblasts</td>
</tr>
<tr>
<td>Bladder</td>
<td>Colon carc (N = 8)</td>
<td>Smooth muscle cells</td>
</tr>
<tr>
<td>Prostate</td>
<td>Mixed tumor of parotid gland</td>
<td>Brain pericytes</td>
</tr>
<tr>
<td>Lung</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placenta</td>
<td>Granulation tissue (N = 4)</td>
<td></td>
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<tr>
<td>Fetal tissue</td>
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*EC, Endothelial cells.
IgG antibodies were purchased from TAGO Inc., Burlingame, California, and from Dakopatts, Copenhagen, Denmark.

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