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Human Endomucin Is an Endothelial Marker

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The identification of novel endothelial markers is important in the study of angiogenesis, and may have potential uses in cancer diagnosis and treatment. We have isolated potential markers of tumor angiogenesis by screening human umbilical vein endothelial cells (HUVECs) treated with tumor conditioned media. Using suppression subtractive hybridization (SSH), we found endomucin, a potential cell surface marker up-regulated in this system. Human endomucin is predicted to encode a 261-aa, 27.5-kDa protein with a transmembrane sequence and multiple glycosylation sites. Northern and *in situ* hybridization studies show that human endomucin expression is largely, if not uniquely, endothelial cell-specific. Human endomucin is present abundantly in highly vascular tissues such as heart, kidney, and lung. It is seen in human aortic endothelial cells (HAECs) as well as in human microvascular endothelial cells (HMVECs). Furthermore, its expression is increased when endothelial cells are proliferating or are stimulated by tumor-conditioned media or specific angiogenic factors such as bFGF (basic fibroblast growth factor) and TNFα (tumor necrosis factor), suggesting that endomucin may have a role in tumor angiogenesis.

Key Words: **endomucin; endothelium; tumor angiogenesis; breast.**

The growth and metastasis of solid tumors is dependent on their ability to initiate and sustain new capillary growth, i.e., angiogenesis (1). Angiogenesis is a complex multistep process which includes endothelial cell proliferation, migration and differentiation into tube-like structures. These steps involve changes in the expression of multiple growth factors, proteases and adhesion molecules in endothelial cells, as well as in supporting cells. Researchers have shown that endothelial cells lining established blood vessels have a very slow turnover time, whereas those lining tumor capillaries undergo rapid proliferation and differentiation. Although much has been discovered about adult angiogenesis, it is unclear whether abnormal angiogenesis such as that occurring in solid tumor growth involves different mechanisms from desirable angiogenesis which occurs in endometrial proliferation or in wound healing (2).

In the past, efforts to identify the differences between the proliferating tumor endothelium and the normal quiescent endothelium have included antibody targeting (3), immunohistochemical analysis of known endothelial adhesion molecules (4), and phage display peptide libraries (5). Differential RNA expression cloning has also been pursued in endothelial cells treated with TPA (6) and in endothelial cells derived from colorectal cancer (7). In order to closely mimic a tumor environment, we have attempted to identify endothelial gene products expressed in response to a mixture of tumor derived growth factors found in tumor conditioned media. Toward this goal, we used a subtraction hybridization method called SSH (suppression subtractive hybridization, 8). In HUVEC (human umbilical vein endothelial cell) populations exposed to tumor conditioned media for 4 h, we have isolated approximately 300 up-regulated and another 300 down-regulated clones (9). One of these differentially expressed genes is human endomucin. In the present report, we show that human endomucin expression is confined to endothelial cells in several tissues and that its expression can be upregulated by growth stimulation and by tumor conditioned medium as well as specific angiogenic factors. These results suggest that human endomucin may be a specific marker of proliferating endothelial cells and may play a role in tumor angiogenesis.

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Human: MELLQVTI LFLLPSI CSSNSTGVLEAANNSLVV . . . . TTTK
Murine: - R - - - A-V - -F- L-NSLCH- EDGKDVQ-D- I P TPAETS - - -

Human:  PSITTPNTESLQKNVVTPTTGTTPKGTI TNELLKMSLMSTAT
Murine: A-V- I- GI V-V. T-  PNK- AD- -P- E- - TKSDVSQT-  -VT-  I N

Human: FLTSKDEGLKATTTDVRKNDSI I S NVTVTSVTLPNAVSTLQ
Murine: S- - TPKHEVGT- -  EGPLR-  E- STMK I  - - PNTPTS-  - N- - - P

Human: SSKPKTETQSSIKTTEIPGSVLQPDASPSKTGTLTSIPVT I PEN
Murine: G-QN- I T-  - . . . . . . . . . . L- . . -  - L- KI - A- PSA . . .SLTTA

Human:  TSQSQVI GTEGGKNASTSATSRSYSSIILPVVIALI VITLSVFV
Murine: HTM- LLQD - -DR -  I-  T- PS- TP - - -- --- -  - - - - -V - - - - L- -T

Human:  LVGLYRMCWKADPGTPENGNDQPQSDKESVKLLTVKTISHE
Murine:  - - - - - -  - I - - - R- - - - - - - - -- - - -- - - - - - - - - - - - - -

Human:  SGEHSAQGKTKN
Murine: - -  - - - - - -  - -  - -
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FIG. 1. Alignment of deduced amino-acid sequence of human endomucin with murine endomucin. Dashes (-) represent aligned identical amino acids; dots (.) represent gaps introduced to maintain the alignment, and amino-acid differences in the murine sequence are indicated.

MATERIALS AND METHODS

Sequence analysis. The sequence of all clones was determined in both directions by automated cycle-sequencing by the UCLA Jonsson Comprehensive Cancer Center sequencing facility. Sequence analysis was performed with the Lasergene Navigator (DNASTAR, Inc., Madison, WI) software package and with searches of the GenBank database using BLASTN. For motif analysis, the following internet websites were used: http://pfam.wustl.edu/hmmsearch.shtml, and http://www.isrec.isb-sib.ch/software/PFSCAN_form.html.

Cell culture. Human umbilical vein endothelial cells (HUVECs) were purchased from Clonetics (San Diego, CA). The cells were plated on tissue culture flasks coated with 1.5% gelatin (Difco, Detroit, MI) and were maintained in endothelial growth media (EGM: endothelial cell growth medium completed with 10 ng/ml hEGF (human epithelial growth factor), 2% fetal calf serum (FCS, Gemini, Calabasas, CA), 1.0 μg/ml hydrocortisone, gentamicin and amphotericin-B (Clonetics). Human aortic endothelial cells (HAECs) and human microvascular endothelial cells (HMVECs) were purchased from Cascade (Portland, OR). For some experiments, cells were rendered quiescent by "starving" in culture in Dulbecco's minimal essential medium (DMEM, Life Technologies, Grand Island, NY) lacking additional supplements. For experiments with specific angiogenic factors, the endothelial cells were grown in DMEM with either bFGF (basic fibroblast growth factor, Chemicon International Inc., Temecula, CA) at 5 ng/ml or TNF-α (tumor necrosis factor alpha, Alexis Corp., San Diego, CA) at 200 units/ml.

The human melanoma line C8161 was obtained from Dr. Barsky (Los Angeles, CA), and the human breast cancer cell line MDA-MB231 from American Tissue Type Culture Collection (Rockville, MD) and maintained on non-gelatinized flasks with DMEM and 10% heat-inactivated FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Carlsbad, CA). The tumor conditioned medium was prepared with confluent cultures of either C8161 or MDA-MB231 as previously described (4). Briefly, the serum-free DMEM media bathing the tumor cells over 48 h was collected, spun, and the supernatant concentrated approximately 5- to 10-fold with Centripreps with a 3000 MW cutoff.

Other cells used in this study included benign human fibroblast CCD-sk-27, benign human liver, benign human lung, human breast cancer Mcf-7 and T47D, human colon cancer Colo-205 and Ls-174t, and human prostate cancer LnCap from ATCC. Human myoepithelial HMS cells were obtained from Dr. Barsky. These cells were all grown in DMEM with 10% FCS, with the exception of HMS which was grown in keratinocyte serum-free medium (K-SFM) supplemented with 50 μg/ml bovine pituitary extract and 5 ng/ml recombinant human epidermal growth factor (GIBCO/BRL, Carlsbad, CA).

Northern analysis. The multi-tissue mRNA blots were purchased from Clontech (Palo Alto, CA). For other blots, total RNA was extracted from cell lines using Trizol (GIBCO/BRL). Twenty micrograms of total RNA was loaded per lane and resolved on 1.2% agarose gels prior to transfer to nitrocellulose membranes, as previously described (9). The human endomucin cDNA probe was labeled by the random primer method (10). All blots were also reprobed for β-actin (GIBCO/BRL) content to verify RNA quantity. Bands for Northern blots were quantitated using a Molecular Dynamic Laser Densitometer (Model PSD1) and an Image Quant Version. 1 software program.

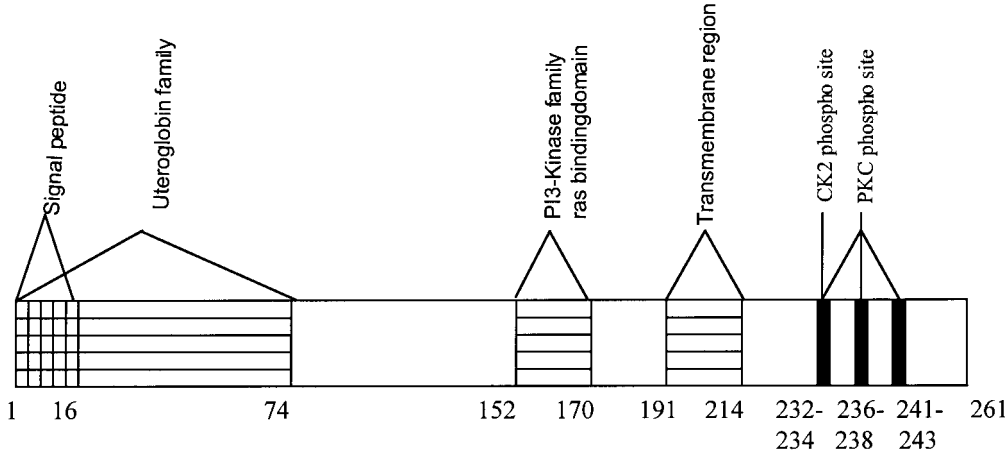

FIG. 2. Structural features of human endomucin. The structure includes a signal peptide sequence (#1–16) and a transmembrane region (#191–214). Motif analysis reveal homology to the uteroglobin family (#1–74) and the PI3 kinase family, ras binding domain (#152–170), one casein kinase II phosphorylation site (#232–235) and three protein kinase C phosphorylation sites (#232–234, #236–238, #241–243).

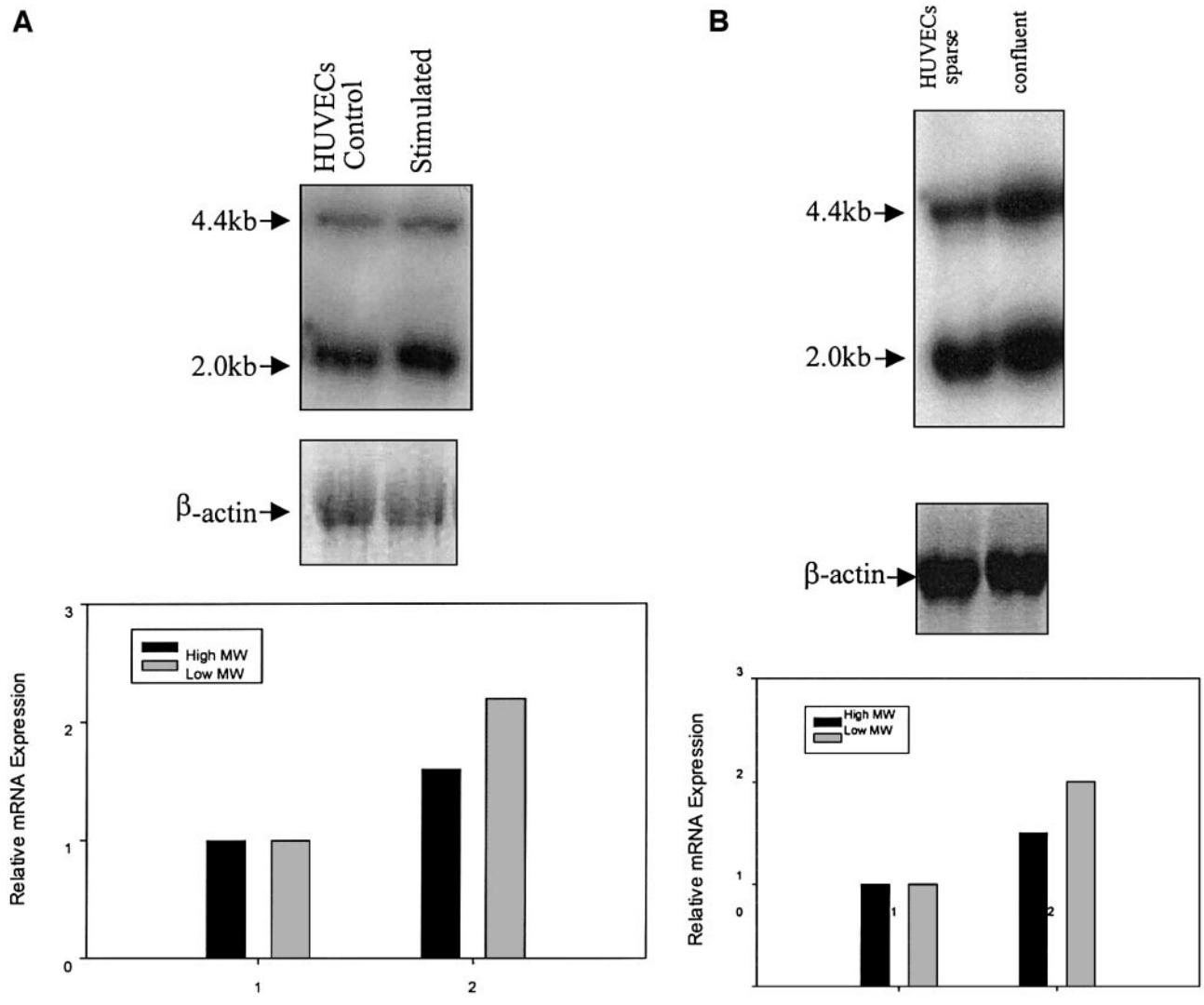

FIG. 3. (A) Induction of human endomucin expression by tumor conditioned media. Control HUVECs (human umbilical vein endothelial cells) were cultured in plain DMEM (lane 1) and stimulated HUVECs in tumor conditioned media (lane 2). Twenty micrograms of RNA was hybridized with human endomucin and β-actin cDNA probes. (B) Regulation of human endomucin expression by proliferation and confluency. HUVECs (human umbilical vein endothelial cells) were cultured in EGM (endothelial growth media). Sparse condition is $<$50% confluency (lane 1) and confluent condition is 80% confluent (lane 2). Twenty micrograms of RNA was hybridized with human endomucin and β-actin cDNA probes.

Human tissue. Human tissue samples were obtained from the UCLA Human Tissue Research Center. Only archival tissue was used, and the identity of the human subjects was removed so as to make the samples untracable. As for all studies involving human tissue, this study was conducted in compliance with the rules of the UCLA Human Subject Protection Committee.

In situ hybridization. Formalin-fixed, paraffin-embedded tissues were sectioned, placed on 3-aminopropyltriethoxysilane-treated slides (GIBCO/BRL), then baked at 60°C for 1 h. The paraffin was removed by incubation in xylene, followed by 100% ethanol. The sections were digested with 40 μg/ml of proteinase K (GIBCO/BRL) for 10 min at 37°C, then washed with PBS. All samples were then fixed for 1 min in 10% buffered formalin, washed with PBS, dehydrated through graded alcohols, and air dried in preparation for hybridization. The probe was labeled with biotin by nick translation according to the manufacturers' instructions (BioPRIME DNA Labeling System, Life Technologies). Unincorporated nucleotides were removed by column chromatography using BioGel P-60 gels (Bio-Rad, Hercules, CA). Double strand probes were heat denatured for 5 min at 100°C prior to hybridization. Hybridization was conducted using the GIBCO BRL In Situ Hybridization and Detection System. Slides were hybridized for overnight at 42°C. After hybridization, the slides were washed in 0.2$\times$ SSC. The signal was detected using streptavidin alkaline phosphatase conjugate and NBT-BCIP (nitroblue tetrazolium, 4-bromo-5-chloro-3-indolylphosphate) substrates. The slides were counterstained with Methyl Green (Sigma, St. Louis, MO), dehydrated through graded alcohols, and mounted with Permount solution (Fisher Scientific, Tustin, CA). Photography was carried out with a Leica DMLS microscope (McBain Instruments, Chatsworth, CA) and a Nikon N6006 camera (Tokyo, Japan).

RESULTS

Analysis of Predicted Sequence

A BLASTN search in the GenBank database reveals that human endomucin is on chromosome 4. The ho-

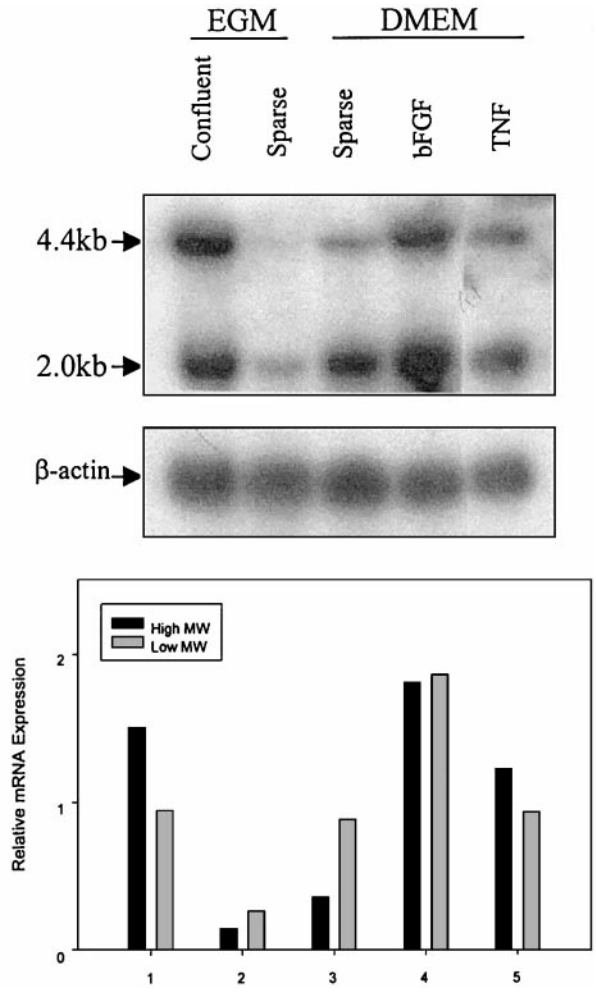

FIG. 4. Induction of human endomucin expression by bFGF (basic fibroblast growth factor) and TNFα (tumor necrosis factor α). HUVECs (human umbilical vein endothelial cells) were cultured in EGM (endothelial growth media) or plain DMEM. Confluent condition is 80% confluency (lane 1), and sparse condition is <50% confluency (lanes 2–3). Sparse HUVECs were exposed to 5 ng/ml bFGF (lane 4) or 200 units/ml TNFα (lane 5). Twenty micrograms of RNA was hybridized with human endomucin and β-actin cDNA probes.

mology of human endomucin to its mouse counterpart (AF060883) is 63% at the nucleotide level and 49% at the peptide level (Fig. 1). From the nucleotide sequence, the predicted peptide has 261 amino acids, and weighs 27.5 kDa. There are 21 strongly basic amino acids, 20 strongly acidic amino acids, 78 hydrophobic amino acids, and 106 polar amino acids. The isolectric point is 7.93, and the peptide as a 0.198 charge at pH 7.0. The peptide has a long extracellular portion (aa 1–190), a transmembrane sequence (aa 191–214), and a cytoplasmic portion (aa 215–261) (Fig. 2). The protein has 36 serine and 42 threonine residues, which make up 30% of total amino acids.

A Profile Scan search reveals a long threonine-rich region spanning from amino acid 33 to 134. There are six N-glycosylation sites, nine protein kinase C phosphorylation sites, four casein kinase II phosphorylation sites, and six N-myristoylation sites. Three of these nine protein kinase C phosphorylation sites and one of the four casein kinase II phosphorylation sites are located in the cytoplasmic portion of the peptide. A Pfam search looking for motif match show some alignment with the following: PI3 (phosphatidylinositol-3) kinase family ras-binding domain (E value 6.6), uteroglobin family (E value 7.5), plant virus coat protein (E value 7.8), bacterial flagellin C-terminus (E value 8.8), and lipoprotein (E value 9.5).

Northern Analysis of Human Endomucin

SSH revealed a gene fragment (AW681211, registered 4/14/00), whose expression is increased in

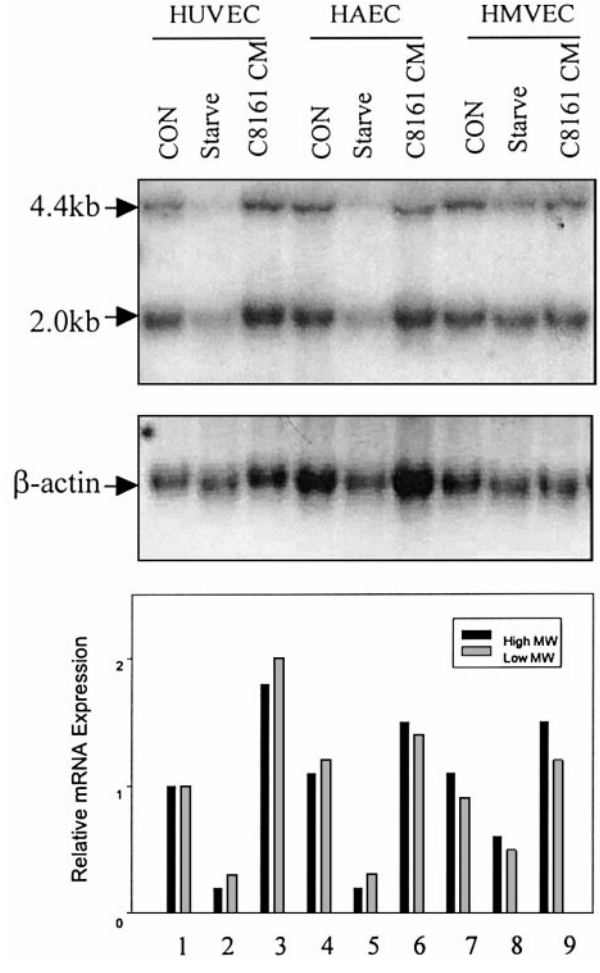

FIG. 5. Presence of human endomucin in different types of endothelial cells. Twenty micrograms of RNA from HUVECs (human umbilical vein endothelial cells), human aortic endothelial cells (HAECs), and human microvascular endothelial cells (HMVECs) were hybridized with human endomucin and β-actin cDNA probes. Control cells were cultured in EGM (endothelial growth media) (lanes 1, 4, and 7). Starved cells were cultured in plain DMEM (lanes 2, 5, and 8). Conditioned medium from the malignant melanoma C8161 was used to stimulate endothelial cells (lanes 3, 6, and 9).

HUVECs treated with tumor conditioned media derived from either melanoma (C8161) or breast cancer (MDA-MB231). While work was in process in our laboratory, a BLASTN search for sequence homology performed in the GenBank database reveals that this sequence is identical to human endomucin (AF205940, registered 6/15/00) and 99% homologous to endomucin-2 (AB034695, registered 11/6/99). Northern analysis confirms that human endomucin expression is upregulated approximately two-fold in HUVECs exposed to tumor conditioned media (Fig. 3A). Two signals corresponding to a 2 kb and a second 4.4 kb are observed to both increase in intensity. Furthermore, human endomucin expression is increased when HUVECS are proliferating and approaching confluency (80% confluency) compared to when they are sparse (<50% confluency) in culture (Fig. 3B). The increase is again approximately twofold, and is seen in both low- and high-molecular-weight bands. We then treated HUVECs to specific angiogenic factors. Stimulation with bFGF increases the expression of human endomucin by approximately two- to threefold, and TNFα by approximately twofold (Fig. 4). When HUVECs are starved, the endomucin transcript level decreases significantly (Fig. 5). The above observations are also seen in other types of endothelial cells including HAECs and HMVECs (Fig. 5). The increase in signal intensity due to exposure to tumor conditioned media is slightly less pronounced in HAECs and HMVECs, in comparison to HUVECs. The decrease in signal intensity secondary to starvation ranges from twofold in HMVECs to threefold in HUVECs and HAECs.

Further Northern studies of human endomucin show that it is highly expressed in heart, kidney, and lung (Fig. 6). The high expression is seen in both 2- and 4.4-kb forms. Human endomucin expression is minimal in peripheral blood leukocytes. When Northern analysis is performed with many different nonvascular cell types, endomucin cannot be detected (Fig. 7). These cell lines include benign types (fibroblast, myoepithelium, liver, and lung) as well as cancer cell lines derived from breast, colon, prostate, and melanoma.

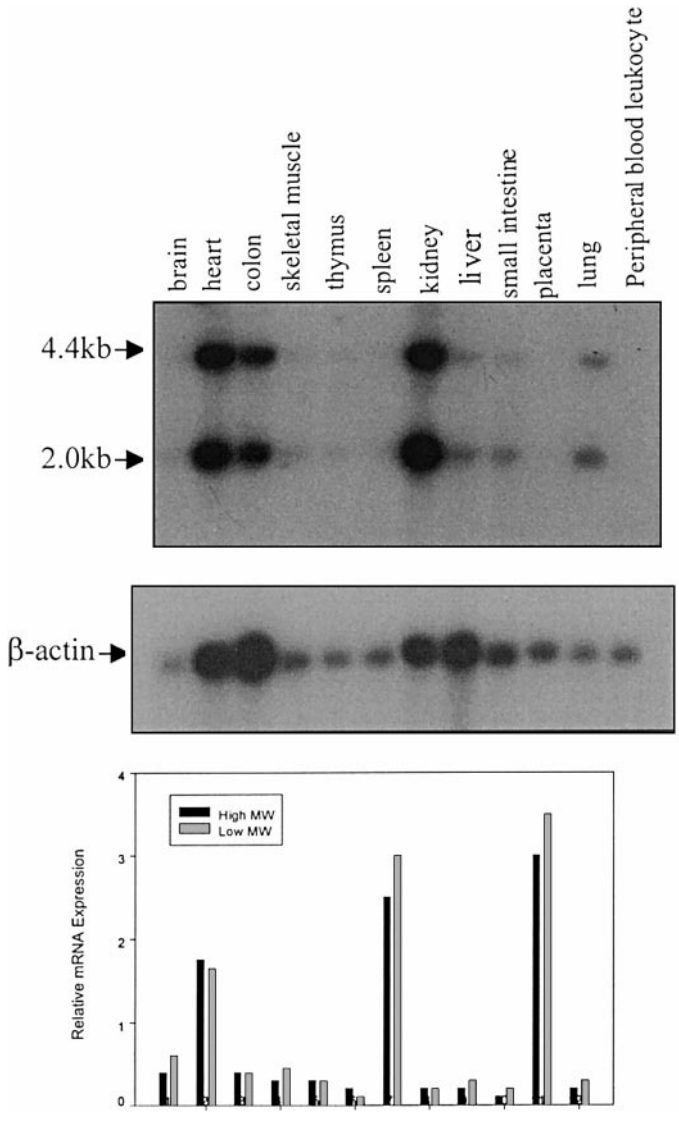

FIG. 6. Presence of human endomucin in different types of human tissues. mRNA multi-tissue blots from Clontech were hybridized with human endomucin and β-actin cDNA probes.

In Situ Hybridization of Human Endomucin

In situ hybridization of human tissues reveal staining in the endothelial cells of blood vessels. This is seen in placenta (Fig. 8A), breast (Fig. 8B), breast cancer (Fig. 8C), and in hemangioma (Fig. 8D).

DISCUSSION

From Northern and *in situ* hybridization studies, it appears that human endomucin expression is largely, if not uniquely, endothelial cell-specific. Unlike mouse endomucin (11), human endomucin is expressed in aortic endothelial cells, as well as in umbilical vein and microvascular endothelial cells. The expression of human endomucin seems to correlate with cellular proliferation or stimulation, as it is down-regulated in starvation and up-regulated by tumor conditioned media. Previously, we have seen that tumor conditioned media from C8161 and/or MDA-MB231 is rich with multiple angiogenic growth factors (12). In this study, we further see that human endomucin expression is increased with exposure to two angiogenic factors bFGF and TNF-α.

Several researchers, including our laboratory, have investigated the difference between surface molecules of the proliferating tumor endothelium from those in the normal quiescent endothelium. One approach toward studying the tumor endothelium involves immu-

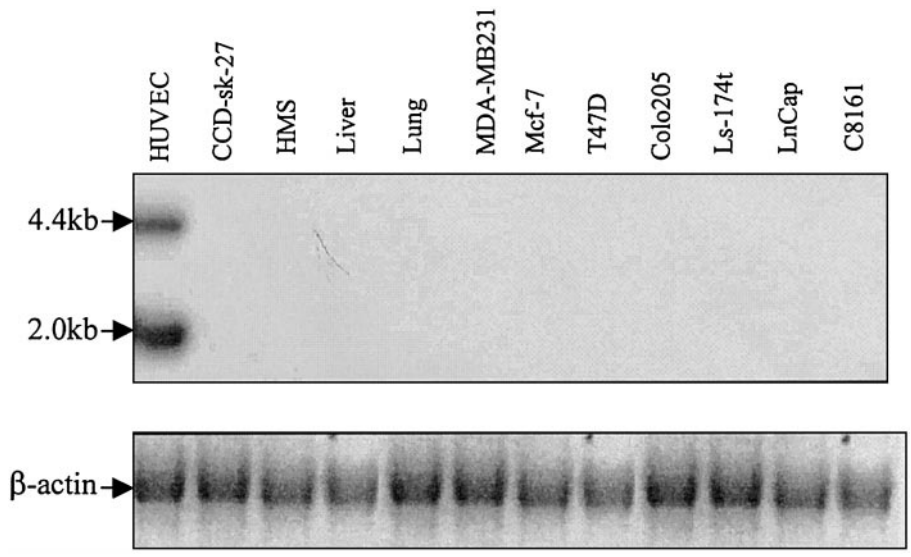

FIG. 7. Absence of human endomucin in nonendothelial cell types. Twenty micrograms of RNA from HUVECs (human umbilical vein endothelial cells) and other cell lines were hybridized with human endomucin and β-actin cDNA probes. Benign human cells include fibroblast CCD-sk-27, myoepithelial HMS, liver, and lung. Malignant human cells include breast cancer MDA-MB231, Mcf-7 and T47D, colon cancer Colo-205 and Ls-174t, prostate cancer LnCap, and melanoma C8161.

nohistochemical analysis of known endothelial adhesion molecules using tumor specimens. These studies have shown that multiple surface molecules are significantly increased in the tumor vasculature. These molecules include E-selectin (4), the $\alpha_v\beta_3$ integrin, VCAM-1 (vascular cellular adhesion molecule), ICAM-1 and -2 (intercellular adhesion molecule), CD 31, CD 34, CD 36, and CD 44 (13). Other investigators have used the antibody targeting approach. This approach has produced multiple candidate markers of the tumor vasculature. These include endoglin which is recognized by the TEC-11 antibody, endosialin which is recognized by the FB5 antibody, the antigen recognized by the EN7/44 antibody, the antigen recognized by the E-9 antibody (14), a truncated form of tissue factor (3), and the fibronectin B-FN isoform (15). Phage display peptide libraries have also been used successfully to characterize tumor blood vessels (5). Differential RNA expression cloning has also been successfully pursued in endothelial cells treated with TPA (6) and in endothelial cells derived from colorectal cancer (7).

In our laboratory, we used SSH to further investigate the molecular mechanisms of tumor angiogenesis by identifying genes that become activated as well as those that become down-regulated when quiescent endothelial cells are exposed to a tumor environment. Although this project utilizes cells in tissue culture, we think that this *in vitro* model does provide an adequate simulation of the tumor environment. Other investigators have used similar methods of differential display to study non-cancer related *in vitro* models of angiogenesis and have found increased expression of important angiogenesis-related genes such as EDG-1 (endothelial differentiation gene, 6) and COX-1 (cyclooxygenase, 16).

The human endomucin molecule shows no significant homology to any known glycoprotein. Based on sequence analysis, human endomucin might be involved in signal transduction. The presence of three protein kinase C phosphorylation sites and one casein kinase II phosphorylation site in the cytoplasmic tail indicates that endomucin might have the capacity to be a signaling molecule. Furthermore, there is some overlap with the PI3 kinase family ras-binding domain (17). Human endomucin also shows some motif alignment with the uteroglobin family. Uteroglobin is a steroid inducible multifunctional protein, secreted by mucosal epithelia in the bronchi, uterus, and prostate. It is thought to be a potent anti-inflammatory protein (18), and may reverse malignant transformation (19). Of note, mammaglobin, a member of this family, has been detected in breast cancer cells (20).

The function of human endomucin is unclear at the present time. Based on its absence in high endothelial venules in lymph nodes, it has been suggested that mouse endomucin may be an anti-adhesive molecule that suppresses leukocyte–endothelial interactions (11). In transfection in vitro experiments, Kinoshita *et al.* (21) recently showed that overexpression of endomucin inhibits the adhesion of HEK293T and NIH3T3 cells to the glass surface of the culture dishes. However, extracellular matrix proteins such as laminin, collagen 1, and fibronectin reversed this effect. In this paper, the reported endomucin-2 differs from human endomucin at a single amino acid (#91). Both human and mouse endomucin are predicted to be highly gly-

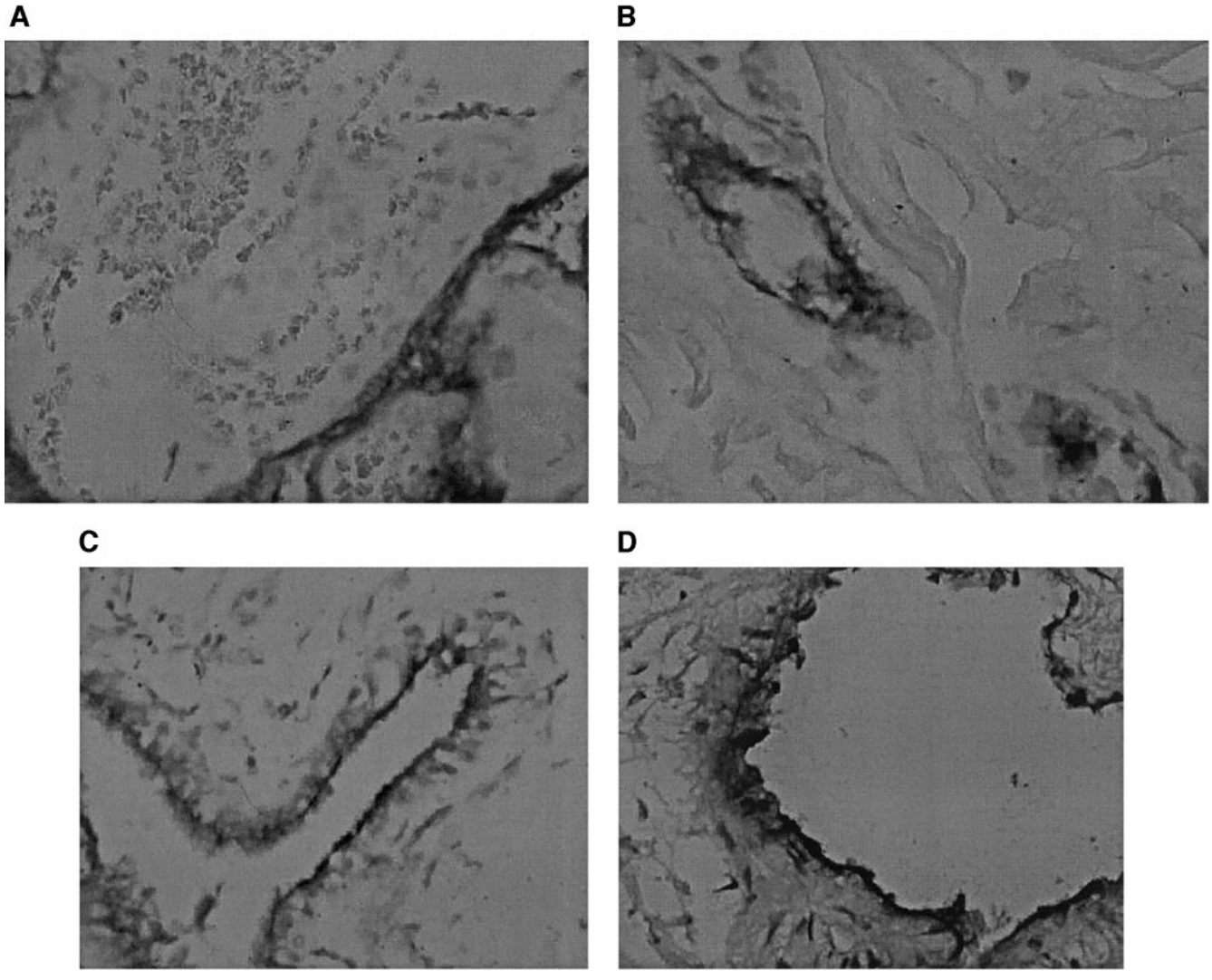

FIG. 8. Presence of human endomucin in the blood vessels of (A) placenta, (B) breast, (C) breast cancer, and (D) hemangioma. *In situ* hybridization was performed as detailed under Materials and Methods.

cosylated membrane proteins. As a membrane protein, human endomucin may have a role in one or more steps of angiogenesis such as endothelial proliferation, migration or differentiation into tube-like structures. It may also mediate the interaction between endothelial cells with other cell types, such as those of the hematopoietic lineage during inflammation, or with cancer cells during metastasis. However, it may be possible that although the expression of human endomucin is altered in this model of angiogenesis, the gene itself does not really play any role in angiogenesis. If this is the case and if the association with endothelial cells remains specific, human endomucin can still be utilized as a marker of angiogenesis, which can potentially be useful as a tumor marker. Furthermore, as a marker, human endomucin can potentially be targeted in the treatment and diagnosis of human disease. Potential utility is seen in many angiogenesis-related diseases including heart disease and stroke, as well as in cancer.

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