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Identification of the S-Endo 1 Endothelial-Associated Antigen

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We have recently described a monoclonal antibody, S-Endo 1, recognizing a molecule constitutively expressed in all types of human endothelial cells. We showed that this protein around 118 kDa and located at the endothelial cell–cell junction presented sequence identity with MUC18 described as a tumor marker in human melanoma. The difference in antibodies immunoreactivity and antigen molecular weight heterogeneity observed between various cell types strongly suggested S-Endo 1 antigen isoforms expression.

Interest in cell surface antigens (Ag) of vascular endothelial cells has stemmed from several lines of research, including studies of blood clotting, lymphocyte homing, inflammation and tumor metastasis (1,2). To explore the pathobiology of human vascular endothelium, we have developed a monoclonal antibody (mAb), called S-Endo 1, directed against cultured human umbilical vein endothelial cells (HUVEC) (3,4). S-Endo 1 Ag is highly expressed in the majority of human endothelial cells of various tissue sections (5). Vessels of different types including arteries, arterioles, veins, veinules, capillaries, high endothelial venules and lymphatic vasculature are positive. But the reactivity of S-Endo 1 is not strictly limited to endothelial cells, since we also detected a faint positivity on smooth muscle cells (SMC), follicular dendritic cells and basal layers (5).

On the basis of its reactivity and compared to the 56 mAbs of the endothelial section of the fifth Workshop (6), S-Endo 1 recognized an apparently novel Ag constitutively expressed on human endothelium. In the present paper, we report the further characterization of the S-Endo 1 molecule, including the amino acid sequence analysis and provide evidence of isoforms expression.

MATERIALS AND METHODS

mAbs. The monoclonal antibodies used in this study were S-Endo 1 (mouse IgG1) raised against HUVEC as previously described (5), MUC18 (culture supernatant, mouse IgG2a), and ST4 (mouse IgG1, Biocytex, Marseille, France).

Cells. Endothelial cells were harvested from human umbilical cord vein as previously described (7). The cells were maintained in RPMI 1640 (Gibco-BRL, Cergy Pontoise, France) containing 20% fetal calf serum (FCS). Human melanoma cell lines Me1-JuSo transfected with MUC18 cDNA (M18SKMe125) or untransfected (SKMe125) were maintained in RPMI 1640 supplemented with 5% FCS. Melanoma cells, IC8 (poorly metastatic clone) and TW12 (highly metastatic clone), were maintained in McCoy 5A medium (Gibco-BRL) supplemented with 10% FCS. The other melanoma cells (Dorier, Beurret, B744, Daudel), colic carcinoma cells (CO115), leukemic cells (Nalm6, pre-B; Raji, B; and CEM30, T cell lines), and EA.hy, endothelial cell line (8), were maintained in RPMI 1640 supplemented with 10% FCS and 1% sodium pyruvate.

Lysates. Confluent cells were washed with PBS and solubilized 20 min at 4°C in RIPA buffer (150mM NaCl, 50mM Tris-HCl, pH8,0, 1% NP40, 1% deoxycholate, 0,1% SDS) containing 1,25mM PMSF, 10mM leupeptine and 5mM d'α_2-macroglobuline. Human frozen placenta was mixed with a polytron homogenizer in the same buffer. All lysates were then centrifuged at 4°C, 100 000 g, for 30 min.

Western blot. Supernatants were fractionated on a 6,7% SDS-polyacrylamide gel, under reducing (8% β_2-mercaptoethanol) or non-reducing conditions and then electrophoretically transferred onto nitrocellulose membranes (Amersham, les Ulis, France). Non specific reactivity was reduced by incubating membrane with 5% milk powder and 0,1% Tween 20 in PBS 2h at 37°C. The nitrocellulose filter was incubated 1h with 10μg/ml of S-Endo 1, washed with 0,1% Tween 20 in PBS and incubated 45 min with 1:5000 horseradish peroxidase anti-mouse (Amersham). The blots were washed and developed with chemiluminescent substrate (Amersham).

Purification and amino acid sequencing. Placenta lysate was precleared by addition of sepharose G protein (Pharmacia, St Quentin Yvelines, France). The supernatant was incubated with S-Endo 1 and immune complexes were collected by centrifugation. After extensive washing, immunoprecipitate was reduced, separated on a 6,7% SDS-polyacrylamide gel,

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blotted onto polyvinyledene fluoride membrane (Bio-Rad, Ivry sur Seine, France) and then stained with Coomassie blue. Immobilized protein was excised and digested *in situ* with trypsin (9). The released peptides were separated with an Applied 140A HPLC system and three peptide peaks were selected for sequencing (Applied 476A). Analysis of the peptide sequences were performed using the GENPRO Protein sequence database.

Immunofluorescene and immunoperoxidase staining. HUVEC plated on glass slides were fixed with 3% paraformaldehyde for 30 min and blocked with 5% FCS for 15 min. S-Endo 1 (10μg/ml) was added for 1 h. After washes, the cells were incubated with 1:100 dilution of FITC labeled $F(ab')_2$ sheep anti-mouse IgG (Silenus, Hawthorn, Australia). Immunoperoxidase staining was realized as previously described (5).

Flow cytometric analysis. Detached cells (M18SKMe125 and SKMe125) were labeled with S-Endo 1 at 4°C for 1h and incubated with FITC labeled anti-mouse under the conditions described above. Fluorescent labeled cells were analysed by flow cytometry using an Epics Profile (Coultronics, Margency, France).

RESULTS AND DISCUSSION

We have previously stated that S-Endo 1 reacted strongly with various human endothelium, including placenta and umbilical cord and with cultured endothelial cells (HUVEC and EA.hy) (6).

The subcellular localization of S-Endo 1 Ag was examined by immunofluorescence staining on unstimulated HUVEC. The Ag was diffuse on membrane but concentrated at cell-cell contact sites (Fig.1).

Structural and biochemical characterization of S-Endo 1 Ag was performed from HUVEC lysates. By western blot analysis (Fig.2), S-Endo 1 detected a single molecule around 118 kDa under non-reducing conditions. No antibody binding occurred under reducing conditions, indicating that at least one disulfide bond was necessary to maintain the conformation of S-Endo 1 epitope.

In view of its tissue distribution, cell-cell localization and biochemical features, S-Endo 1 Ag seems to be a novel molecule. Since HUVEC are not easily cultured in large amount, we have chosen to isolate and characterize S-Endo 1 Ag from human placenta, an important source of endothelial cells. Protein sequencing of NH2-terminal and tryptic peptides of the 118 kDa protein were performed after immunoprecipitation of human placenta lysate. A sequence identity was revealed between S-Endo 1 Ag and MUC18. As shown in Fig.3, peptide sequences completely matched the deduced amino acid positions 27–40, 99-113, 242-262 of the MUC18 molecule (10). MUC 18 was first described as a marker of human melanoma progression (11,12). This cell surface

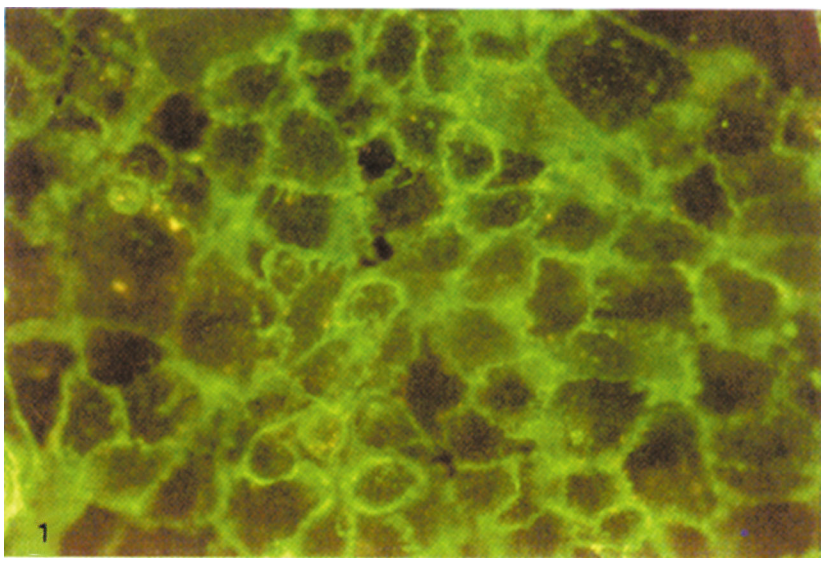

FIG. 1. Immunolocalization of S-Endo 1 antigen at the cell–cell contact. Immunofluorescence labeling was performed on unstimulated HUVEC.

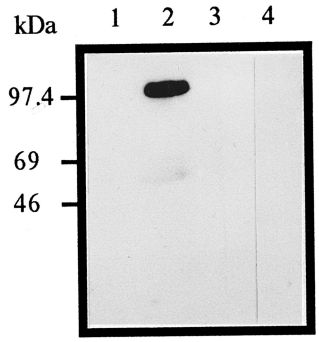

FIG. 2. Western blotting of S-Endo 1 antigen expressed on endothelial cells. HUVEC lysate was subjected to 6.67% SDS–PAGE under nonreducing conditions (lane 1) and reducing conditions (lane 2) followed by immunoblotting revealed with S-Endo 1. Raji, nonexpressed S-Endo 1 Ag, was used as negative control.

glycoprotein, located at the cell-cell contact, is a member of the immunoglobuline superfamily (IgSF), probably involved in adhesion. (13)

To confirm that S-Endo 1 Ag and MUC18 are the same molecule, human melanoma cell line (SKMe125) transfected with MUC18 cDNA was examined for its immunoreactivity with S-Endo 1. Flow cytometry analysis showed that only transfected cells (M18SKMe125) were S -Endo 1 positive (Fig.4) and western blotting of M18SKMe125 cell lysates revealed with S-Endo 1 demonstrated a 118kD protein band (data not shown). Western blot analysis of HUVEC lysates probed with S-Endo 1 and MUC18 mAb revealed the same reactivity pattern in Fig.5 (use of culture supernatant for MUC18 mAb explains the weaker intensity compared to use of purified S-Endo 1). All the results unambiguously show that S-Endo 1 Ag and MUC18 are the same molecule.

In normal tissue, MUC 18 Ag was reported to be strongly expressed on smooth muscle cells but restricted on endothelium to capillaries and high endothelial venules (14), while our preceding reports (5,6) show a strong S-Endo 1 staining in all types of human endothelium whatever the vessel calibers. In particular, MUC18 mAb failed to detect the Ag on umbilical cord vein (14,15), while S-Endo 1 did (Fig.6). This observation suggests that the two mAbs recognized different epitopes; the epitope recognized by MUC 18 could be inaccessible or absent in some tissue. These results raise the possibility that several biochemically closely related isoforms of the Ag exist.

Therefore, we analysed the migratory behavior of the Ag extracted from different cells by

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1:MGLPRLVCAFLLAACCCCPRVAGVPGEAEQPAPELVEVEVGSTALLKCGLSQSQGNLSHVDWFSVHKEKRTLIFRVRQGQ
                                EAEQPAPELVEVEV

81:GQSEPGEYEQRLSLQDRGATLALTQVTPQDERIFLCQGKRPRSQEYRIQLRVYKAPEEPNIQVNPLGIPVNSKEPEEVAT
                              GATLALTQVTPQDER

161:CVGRNGYPIPQVIWYKNGRPLKEEKNRVHIQSSQTVESSGLYTLQSILKAQLVKEDKDAQFYCELNYRLPSGNHMKESE
                                                                                    E

241:VTVPVFYPTEKVWLEVEPVGMLKEGDRVEIRCLADGNPPPHFSISKQNPSTREAEEETTNDNGVLVLEPARKEHSGRYEC
    VTVPVFYPTEKVWLEVEPVG

321:QAWNLDTMISLLSEPQELLVNYVSDVRVSPAAPERQEGSSLTLTCEAESSQDLEFQWLREETDQVLERGPVLQLHDLKRE

401:AGGGYRCVASVPSIPGLNRTQLVKLAIFGPPWMAFKERKVWVKENMVLNLSCEASGHPRPTISWNVNGTASEQDQDPQR

481:LSTLNVLVTPELLETGVECTASNDLGKNTSILFLELVNLTTLTPDSNTTTGLSTSTASPHTRANSTSTERKLPEPESRGV

561:VIVAVIVCILVLAVLGAVLYFLYKKGAAVQALREAGDHAAPVS
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FIG. 3. Comparison of amino acid sequences between reported MUC18 and purified S-Endo 1 antigen. Three fragments of trypsin digested S-Endo 1 peptides were subjected to internal and NH2-terminal sequencing. The results are shown in the *lower part* of the sequence. The amino acid sequence of the Muc18 protein was deduced from the nucleotide sequence of the Muc18 cDNA corrected.

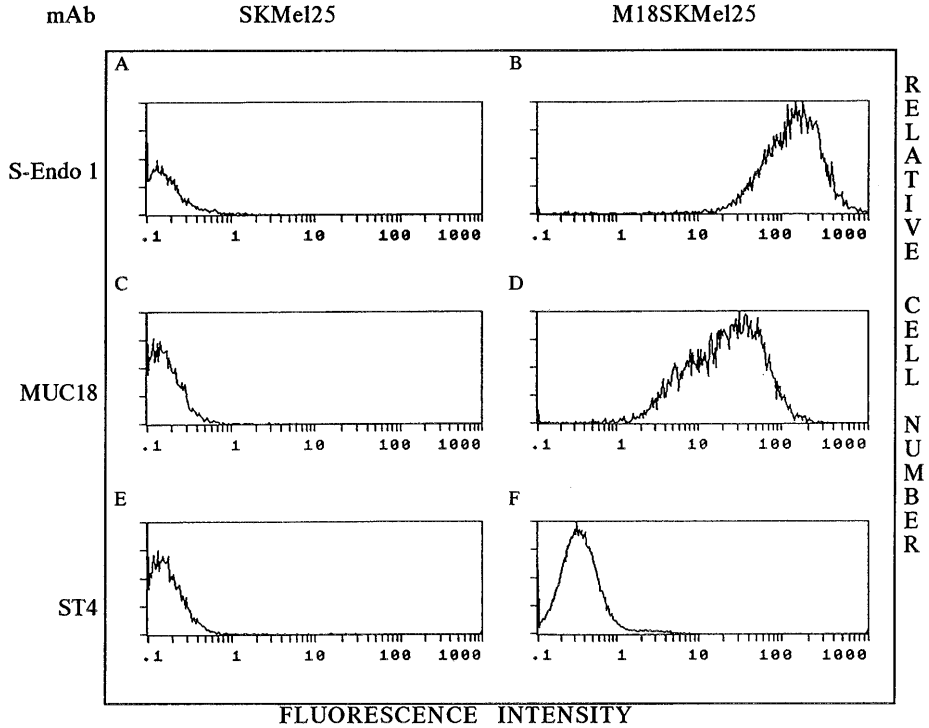

FIG. 4. Flow cytometric analysis of S-Endo 1 reactivity on cDNA MUC18 transfected and untransfected melanoma cell lines S-Endo 1 only reacted with trasfected melanoma cells (B) like MUC18 mAb (D), used as positive control. ST4, irrelevant mAb was unreactive with the two cell types (E, F).

western blot analysis probed with S-Endo 1 (Fig.7). In HUVEC, S-Endo 1 detected a 118 kDa protein. In some experiments, a minor band around 100 kDa was also revealed. In endothelial cell line (EAhy), the two bands (118 kDa and 100 kDa) were always detected in equal amounts. Placenta and umbilical cord showed a polydisperse band. When used in immunoblotting of melanoma cell lines, S-Endo 1 detected a ∼118 kDa protein in TW12, Daudel, B744 and a ∼122 kDa protein in Beurret, IC8 and Dorier. B744 presented an additional band around 180 kDa. Other types

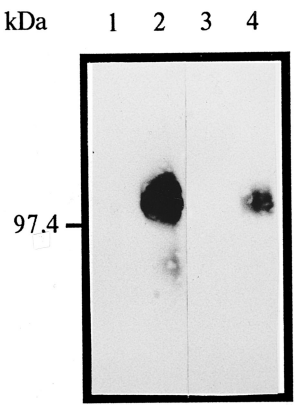

FIG. 5. Comparison of S-Endo 1 and MUC18 antibody reactivity on HUVEC. A western blotting was performed on HUVEC and Raji (used as negative control). Raji (lanes 1 and 3) and HUVEC (lanes 2 and 4) were fractionated on 6.67% SDS–PAGE under nonreducing and detected with S-Endo 1 (lanes 1 and 2) and MUC18 (lanes 3 and 4).

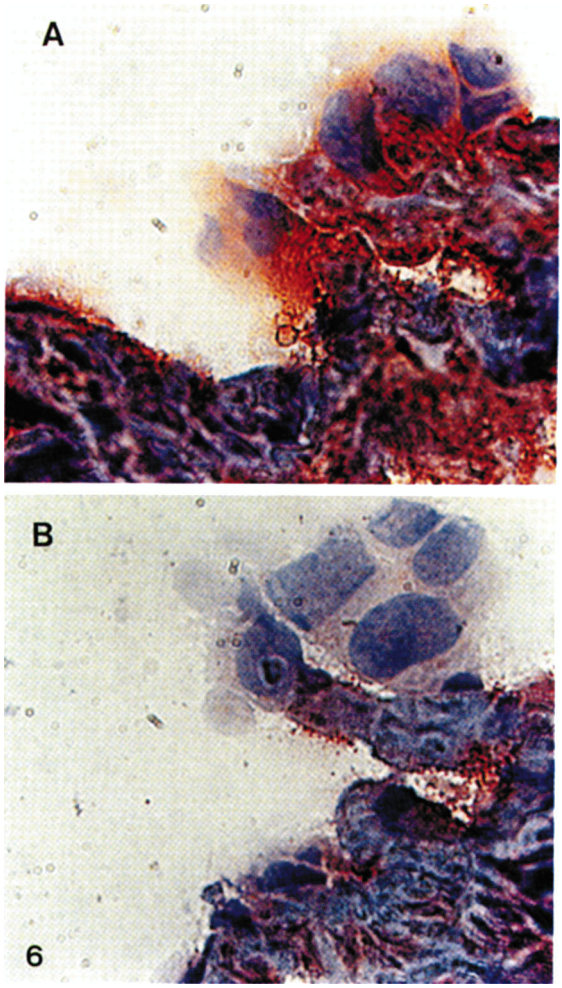

FIG. 6. Comparison of S-Endo 1 and MUC18 antibody reactivity on umbilical vessel cord. Frozen and acetone-fixed cryostat sections of human umbilical cord were stained with S-Endo 1 or MUC18 by an indirect immunoperoxidase technique. S-Endo 1 strongly reacted with vessel of umbilical cord(A) while MUC 18 did not (B).

of tumor were also studied. A ~125 kDa and a ~100 kDa protein were revealed on CO115, and a ~122 kDa protein on CEM30 and Nalm6. In contrast to preceding reports (13), we found that MUC18 is not restricted to melanoma among tumoral cells and demonstrated for the first time that the Ag is also expressed on other tumor types (colic carcinoma cells) as well as on leukemic cell lines (T and pre-B cell lines).

Taken together our results demonstrated that the molecular weight of S-Endo 1 Ag varied from 125 kDa to 118 kDa among the different cell types examined with additional bands (180 kDa and 100 kDa). It is well known that some Ig SF members and other cell adhesion molecules appeared in several isoforms (16,17). As described for several Ig-like CAMs, including MAG (18), CEA (19) and N-CAM (20), these results can represent transcriptional (such as alternative splice) or post-transcriptional modifications (glycosylation), which could alter CAM interaction. A number of descriptive studies have analysed differences in glycoprotein expression patterns of endothelial cells showing organ-specific and caliber-specific characteristics (21). The polydisperse band on placenta and umbilical cord, non observed on cultured endothelial veins (Fig.7), may reflect a

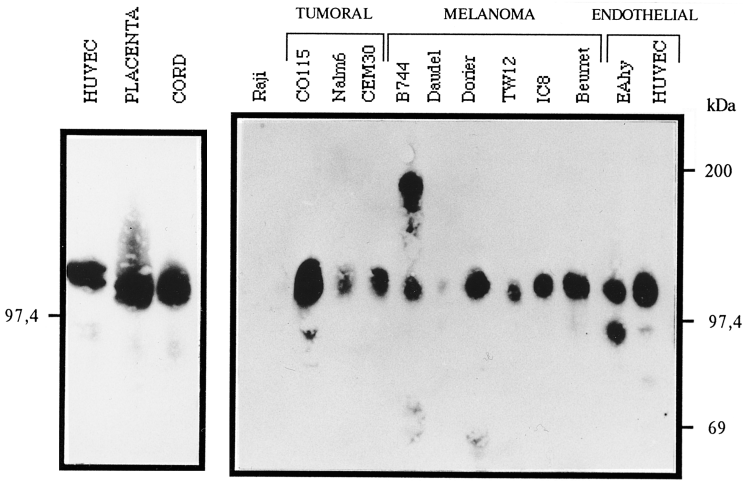

FIG. 7. S-Endo 1 antigen isoforms. On Western blot, the different lysates of cells were separated on 6.67% SDS–PAGE under nonreducing conditions and revealed with S-Endo 1. Raji were used as negative control.

cellular heterogeneity and more precisely an endothelial diversity since arteries, arterioles, veins, veinules and capillaries are present in these tissues. As the cell culture models (from large vessel) are not the answer, Ag expression in vascular endothelium from vessels of different sizes and from different anatomical compartments is worth investigation. Interestingly S-Endo 1 Ag molecular weight variability was observed among different clones established from the same human melanoma cell line, IC8 and TW12, both coming from Beurret and presenting different metastatic power (low and high respectively). IC8 and TW12 may differ in their oligosaccharide composition. This possibility is more plausible when considering that alteration of cell surface glycoconjugates has been associated with invasiveness and metastatic capacity (22).

Present in all types of human endothelium, this new member of Ig SF may play an important role in a wide variety of vascular adhesive processes. Given that our results strongly suggested S- Endo 1 Ag isoforms expression, it will be interesting to investigate if the different isoforms define tissue specific adhesive potential depending on vascular sites.

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