Characterization of human vascular endothelial cadherin glycans

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The glycosylation pattern of human vascular endothelial cadherin (VE-cadherin), purified from cultured human umbilical cord vein endothelial cells, was analyzed. VE-cadherin was metabolically radiolabeled with D-[6-³H]glucosamine, isolated by immunoprecipitation, purified by SDS-PAGE and in-gel digested with endoproteinase Asp N. Oligosaccharides were sequentially released from resulting glycopeptides and analyzed by chromatographic profiling. The results revealed that VE-cadherin carries predominantly sialylated diantennary and hybrid-type glycans in addition to some triantennary and high mannose-type species. Highly branched, tetraantennary oligosaccharides were found in trace amounts only. Immunohistochemical labeling of VE-cadherin and sialic acids displayed a codistribution along the intercellular junctions in endothelial cells of human umbilical arteries, veins, and cultured endothelial monolayers. Ca²⁺-depletion, performed on cultured endothelial cells, resulted in a reversible complete disappearance of VE-cadherin and of almost all sialic acid staining from the junctions. Sialidase treatment of whole cells caused a change of VE-cadherin immunofluorescence from a continuous and netlike superstructural organization to a scattered inconsistent one. Hence, cell surface sialic acids might play a role in VE-cadherin organization.

Key words: VE-cadherin/carbohydrate analysis/ oligosaccharides/VE-cadherin-superstructure

Introduction

Intercellular junctions of the cardiovascular endothelium consist of various integral membrane proteins able to directly mediate interendothelial adhesion: (1) Ca²⁺-dependent cadherins consisting of vascular endothelial (VE) cadherin, VE-cadherin-2 (Telo *et al.*, 1998) and N-cadherin (Salomon *et al.*, 1992; Navarro *et al.*, 1998); (2) the platelet endothelial cell adhesion molecule 1 (PECAM-1) belonging to the Ca²⁺-independent immunoglobulin superfamily; (3) the α_2/β_1- and α_5/β_1-integrins; and finally (4) occludin, a component of the tight junctions (for review, see Dejana *et al.*, 1995; Dejana, 1996; Schnittler and Feldmann, 1998).

The vascular endothelial cadherin (VE-cadherin) is endothelium specific (Suzuki *et al.*, 1991; Lampugnani *et al.*, 1992) and

belongs to endothelial adherens junctions that are commonly found in endothelium of all locations *in situ* and in culture (Simionescu *et al.*, 1975; Simionescu *et al.*, 1976; Franke *et al.*, 1988, 1989). Ca²⁺-dependent cadherins are integral membrane proteins that consist of a large amino-terminal extracellular domain (ectodomain) with five repeats, one membrane spanning domain and a short carboxy-terminal cytoplasmic tail (Takeichi, 1995). The extracellular domains probably occur as parallel "strand dimers" by which the terminal repeat of the extracellular domain binds to cadherins of adjacent cells (antiparallel "adhesion dimers") forming a zipper-like cell-to-cell connection (Shapiro *et al.*, 1995a,b). Cadherins are linked to cytoplasmic actin filaments via α-, β-, and γ-catenin (plakoglobin) (reviewed in Jou *et al.*, 1995; Klymkowsky and Parr, 1995; Takeichi, 1995; Aberle *et al.*, 1996; Yap *et al.*, 1997). The cadherin/catenin complex of the epithelium and endothelium contributes to tissue organization and maintenance in developing and adult organisms (for review, see Dejana *et al.*, 1995; Klymkowsky and Parr, 1995; Takeichi, 1995; Aberle *et al.*, 1996; Yap *et al.*, 1997). In endothelial cells, the cadherin/catenin complex seems to be crucially involved in certain physiological and pathophysiological reactions such as regulation of permeability, extravasation of leukocytes and tumor cells, as well as cell migration and resistance to fluid shear stress (Lampugnani *et al.*, 1992, 1995, 1997; Dejana, 1996; Del Maschio *et al.*, 1996; Feldmann *et al.*, 1996; Rabiet *et al.*, 1996; Levalle *et al.*, 1997; Schnittler *et al.*, 1997; Moll *et al.*, 1998; Schnittler and Feldmann, 1998).

The polypeptide chain of VE-cadherin comprises seven potential N-glycosylation sites (Suzuki *et al.*, 1991; Breviario *et al.*, 1995), all localized in the VE-cadherin ectodomain. First evidence for a contribution of glycan chains to cadherin function in epithelial cells was obtained by Yoshimura and co-workers (Yoshimura *et al.*, 1996) who demonstrated that ectopically in murine melanoma B16-hm cells expressed (β1–4)-*N*-acetylglucosaminyltransferase (GnT-III) caused an altered glycosylation of E-cadherin that in turn was associated with a reduced protein turnover rate, increased expression of E-cadherin at the intercellular junctions and a reduced metastatic capacity of the cells (Yoshimura *et al.*, 1995, 1996). This indicates that glycosylation of E-cadherin is involved in the organization of functional competent intercellular junctions but a structural characterization of the glycosylation pattern of any native cadherin has not yet been performed.

With respect to the importance of VE-cadherin participation in certain endothelial mediated mechanisms, the aim of this study was to characterize the overall glycosylation pattern of VE-cadherin in order to enable further functional investigations. Here we show that (1) VE-cadherin carries predominantly sialylated complex and hybrid-type glycans, (2) sialic acids can be largely colocalized with VE-cadherin molecules at the interendothelial junctions of endothelial cells *in situ* and in culture, and (3) cell surface sialic acids seem to be important for the structural organization of VE-cadherin clusters.
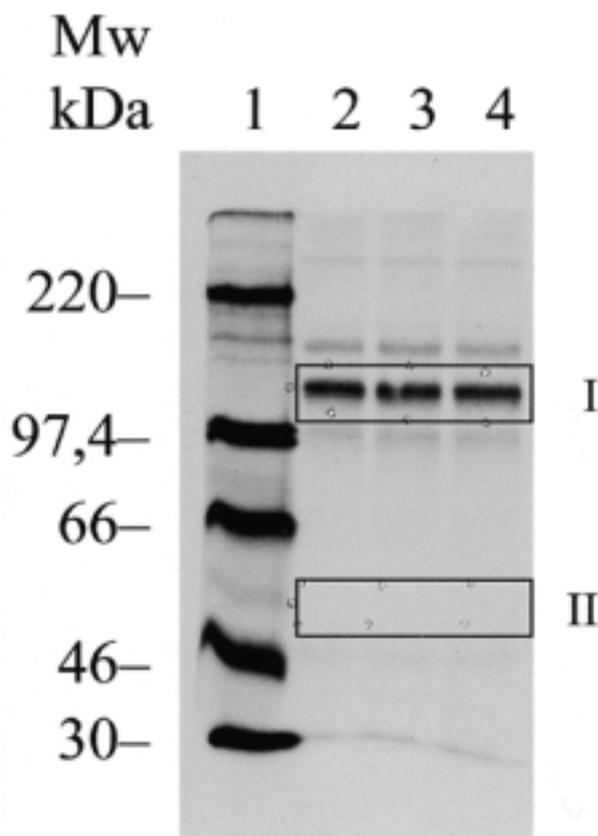
Fig. 1. Purification of human VE-cadherin. VE-cadherin obtained from human umbilical vein endothelial cell (HUVEC) extracts after *in vivo* labeling with [6-³H]glucosamine and immunoprecipitation was purified by semipreparative SDS-PAGE. Radioactive protein bands were visualized by fluorography. Gel segments containing VE-cadherin (I) were excised and subjected to carbohydrate analysis. For background control, equivalent gel segments (II) were similarly worked up. Lane 1, mass marker proteins; lanes 2–4, VE-cadherin after immunoprecipitation.

Results

Isolation of VE-cadherin

Cultured human endothelial cells were labeled with [6-³H]glucosamine and subsequently extracted with 1% Triton X-100 in buffer as described in *Material and methods*. The use of 1% Triton X-100 causes a release of about 70–80% of the cadherin/catenin complex (Schnittler *et al.*, 1997) that can be immunoprecipitated from a 8000 × *g* supernatant using a monoclonal VE-cadherin antibody. Precipitated protein was subjected to SDS-PAGE, and fluorography of the gel displayed one main band at MW of 135 kDa and few weaker labeled bands (Figure 1). Part of the precipitate was subjected to Western blot analysis using a pan cadherin antibody known to react with a short carboxyterminal sequence of classical cadherins. This antibody stained a single band exactly at the same MW as the main radiolabeled band (data not shown).

Isolation of glycopeptides

Since SDS displayed several additional, radiolabeled protein bands, immunoprecipitated VE-cadherin samples were preparatively separated by SDS-PAGE, and bands containing VE-cadherin were excised (I in Figure 1). In order to avoid loss of

material when eluting the whole glycoprotein, an in-gel proteolytic digestion was performed, after which radioactive glycopeptides could be easily eluted from the gel pieces. From the known amino acid sequence of the glycoprotein (Suzuki *et al.*, 1991; Breviario *et al.*, 1995), it could be deduced that treatment with trypsin would generate a glycopeptide resistant to peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase F (PNGase F) (Tarentino *et al.*, 1985). Therefore, Asp-N endoproteinase from *Pseudomonas fragi* mutant was used instead. In total, a glycopeptide preparation was obtained comprising about 10^5 cpm of incorporated ³H radioactivity. As a control, unstained gel segments (II in Figure 1) were equally treated and eluted. However, no radioactivity could be detected in the respective supernatants.

Liberation and fractionation of glycans

Western blot analysis, using a VE-cadherin monoclonal antibody, of endothelial cells treated with PNGase F and endo-β-*N*-acetyl-glucosaminidase H (endo H) revealed the presence of both endo H–sensitive and endo H–resistant N-glycans. Treatment with PNGase F resulted in a shift of the VE-cadherin band from about 135 kDa to about 90 kDa (arrow) in agreement with the expected molecular mass calculated from its amino acid sequence (Figure 2, lane 3). The second band (dotted line) is assumed to represent a degradation product. Incubation with endo H also led to a small but significant shift in the electrophoretic mobility of this glycoprotein (Figure 2, lane 5). Therefore, isolated glycopeptides were first treated with endo H. Oligosaccharides released were separated from residual glycopeptides by reverse-phase (RP-) HPLC. Endo H-resistant glycopeptides were incubated with PNGase F. The resulting reaction mixture was again subjected to RP-HPLC to isolate free glycans. Approximately 16% and 73% of total radioactivity were released by the two enzymes. Residual 11% of radioactivity, still eluting in the peptide fraction, could be shown to comprise exclusively *N*-acetylglucosamine (GlcNAc) and no *N*-acetylgalactosamine (GalNAc) and are, therefore, assumed to result from the innermost GlcNAc residue(s) remaining bound to the peptide(s) after endo H cleavage (Kobata, 1979). After reduction, oligosaccharide alditols were fractionated by anion-exchange HPLC. The results revealed that the majority of human VE-cadherin glycans carry negative charges. Complex type species, released by PNGase F, comprised neutral glycans (F0, 7% of radioactivity) in addition to species with one (F1, 49%), two (F2, 38%), or three (F3, 5%) negatively charged residues (Figure 3A). Oligosaccharides released by endo H, representing oligomannosidic or hybrid-type glycans (Kobata, 1979), similarly carried predominantly (75%) one negative charge (data not shown). Treatment with sialidase from *V.cholerae* or mild acid hydrolysis converted all charged species into neutral compounds demonstrating that the negative charge was exclusively conferred by sialic acid. In the case of PNGase F–sensitive oligosaccharide alditols, charged glycans were separated by preparative anion-exchange HPLC and individually digested with α2,3-specific sialidase from Newcastle disease virus. Monosialylated species could not be degraded and, thus, solely contained α2,6-linked sialic acid, whereas di- and trisialylated glycans both contained one (or two) α2,6-linked sialic acid residue(s) in addition to α2,3-bound sialic acid (Figure 3B–D). For further characterization, all glycans were completely desialylated.

Characterization of glycans

Since the majority of endo H–sensitive glycans carried one sialic acid residue, they could be assumed to represent predominantly hybrid-type species. This was corroborated by high-pH anion-
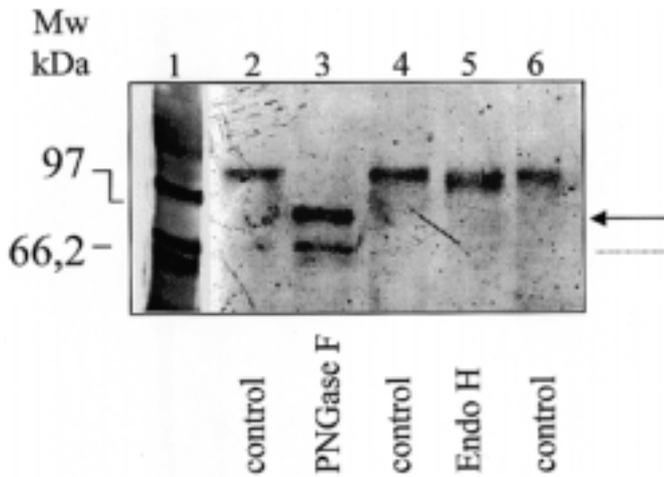
Fig. 2. Western blot analysis of human VE-cadherin. HUVEC extracts were subjected to SDS gel electrophoresis and Western blotting before (lanes 2, 4, and 6) and after treatment with PNGase F (lane 3) or endo H (lane 5). VE-cadherin bands were visualized by specific antibodies. Lane 1, mass marker proteins. The arrow indicates the molecular mass calculated from its amino acid sequence (lane 3). The dotted line indicates an assumed degradation product (lane 3).

Fig. 3. Fractionation of PNGase F-released oligosaccharides from human VE-cadherin by anion-exchange HPLC. (**A**), oligosaccharide alditols obtained after treatment of endo H-resistant glycopeptides with PNGase F, RP-HPLC and reduction were subjected to anion-exchange HPLC using a Mikropak AX-10 column (4.6 × 250 mm) and a linear gradient of 0–300 mM potassium phosphate, pH 4.4, within 60 min. Fractions (400 µl) were collected at 1 ml/min and monitored for radioactivity. Fractions (F0 –F3) were pooled as indicated by brackets. (**B–D**) Rechromatography of F1- (**B**), F2- (**C**), and F3- (**D**) derived glycans after treatment with α2,3-specific sialidase from Newcastle disease virus under similar conditions. Numbers (0–4) with arrows indicate the elution volumes of standard oligosaccharides with 0–4 sialic acid residues. * in (**C**) and (**D**), released sialic acid.

exchange chromatography (HPAEC) of the desialylated glycans (Figure 4A). Only species H3 (14% of radioactivity within this fraction) coeluted with an oligomannosidic standard oligosaccharide (OM9), whereas the elution volumes of the two major components, H1 and H2 (62% and 24%), did not correspond to those of oligomannosidic standards, but to two hybrid-type standard glycans, M_{III} and M_{IV} (Geyer *et al.*, 1984), respectively (for detailed structures, see Figure 4 caption). To further prove their identity, half the amount of endo H–sensitive compounds obtained was digested with α-mannosidase from jack beans. Rechromatography of the resulting products by HPAEC demonstrated that two products were formed eluting at the positions of authentic ManGlcNAcOH and GalGlcNAcMan$_2$GlcNAcOH (Figure 4B). Digestion of the second half of the sample with β-galactosidase (Figure 4C) resulted in a shift of the elution volumes of the two hybrid-type species corresponding to the loss of one galactosyl residue each whereas the elution position of the oligomannosidic glycan H3 remained unchanged. Therefore, it may be assumed that the two major oligosaccharide alditols, H1 and H2, represent hybrid-type species with one *N*-acetyllactosamine antenna and two or three α-linked mannosyl residues. From their sensitivity towards endo H, it may be further concluded that the terminal mannosyl residue present in H1 glycans is α1,3-linked (Kobata, 1979). The minor component H3, on the other hand, represented an oligomannosidic glycan with nine Man residues.

Neutral complex type glycans, obtained after individual desialylation of isocharged species, were chromatographically characterized by HPAEC (Figure 5) and gel filtration using a Bio-Gel P-4 column (data not shown). Although determination of chromatographic parameters does not allow a structural assignment *a priori*, comparison with the elution volumes of a set of appropriate oligosaccharide standards in at least two different chromatographic systems gives reliable results (Liedtke *et al.*, 1997). The glucose units obtained from internal calibration with isomaltooligosaccharides were compared with those of authentic fucosylated di-, two isomers of tri-, tetra-, and bisected dianten-
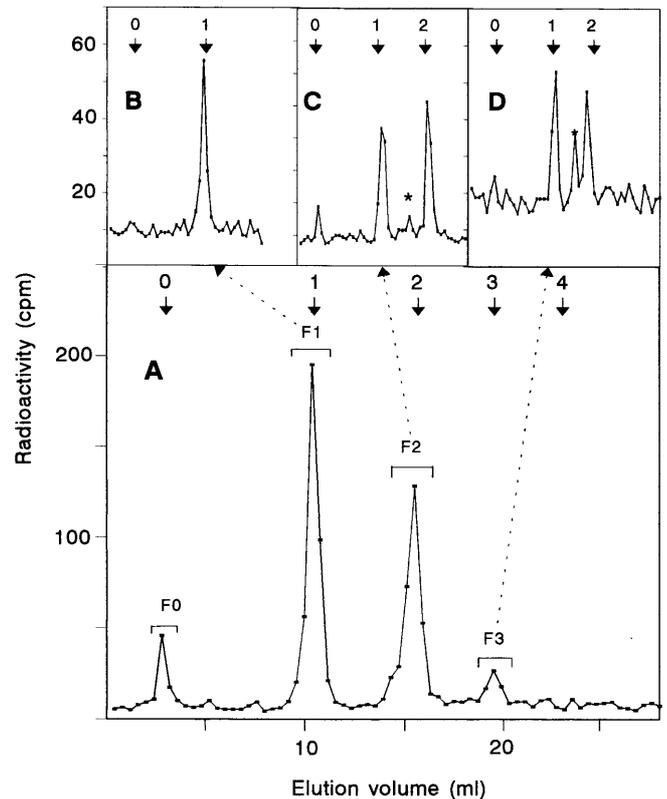
nary standard oligosaccharide alditols. The majority of glycans thus identified (Figure 5A–D; fractions F01, F11, F13, F21, F31, F32) represented fucosylated species with two, three, or four *N*-acetyllactosamine antennae in addition to a few species not coeluting with any of these standard glycans (fractions F02, F12). This assumption was supported by gel filtration data which suggested the presence of incomplete triantennary species lacking one (or two) galactosyl residue(s). To further substantiate these assignments, the four fractions of complex type glycans as well as the respective oligosaccharide standards were treated with β-galactosidase from *D.pneumoniae*. Resulting products were again analyzed by HPAEC and, in part, also by gel filtration. As shown in Figure 5E–H, compounds identified as fucosylated di-, tri-, and tetraantennary species coeluted with the respective agalacto oligosaccharide standards after β-galactosidase treatment. Species F02 and F12, formerly eluting at 4.62 and 4.44 glucose units (Figure 5A,B), now coeluted with triantennary agalacto oligosaccharide standards (Figure 5E,F) and thus may be assumed to comprise fucosylated triantennary species with one (or two) incomplete antenna(e). Differentiation between the possible isomeric forms, however, was not possible. The elution position of species with 2.9 glucose units (Figure 5A) did not
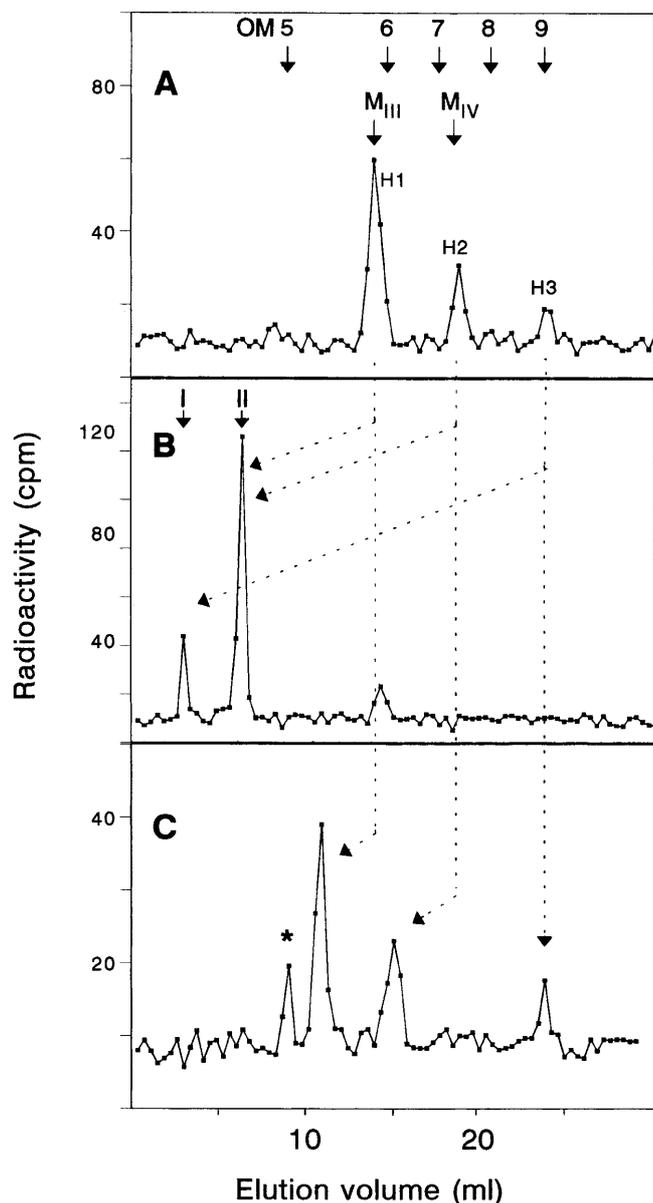
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Fig. 4. Chromatographic profile of desialylated endo H–sensitive glycans from human VE-cadherin. (**A**) Oligosaccharide alditols obtained after treatment of proteolytic glycopeptides with endo H, RP-HPLC, reduction and enzymatic desialylation were separated by HPAEC on a CarboPak PA-100 column (4 × 250 mm) using a gradient of 10–30 mM sodium acetate in 80 mM NaOH within 70 min. Fractions (380 μl) were collected at 1 ml/min and monitored for radioactivity. (**B**) Desialylated endo H–sensitive glycans after treatment with α-mannosidase; (**C**) desialylated endo H–sensitive glycans after treatment with β-galactosidase from *D.pneumoniae*. Numbers (OM5-9) with arrows indicate the elution volumes of oligomannosidic oligosaccharide standard alditols Man$_{5-9}$GlcNAcOH; M$_{III}$, M$_{IV}$, elution volumes of hybrid-type oligosaccharide standard alditols Galβ4GlcNAcβ2Manα3[Manα3Manα6]Manβ4GlcNAcOH and Galβ4GlcNAcβ2Manα3[Manα3(Manα6)Manα6]Manβ4GlcNAcOH, respectively; I, II, elution volumes of Manβ4GlcNAcOH and Galβ4GlcNAcβ2Manα3Manβ4GlcNAcOH. * in (**C**), unidentified product.

change after digestion with β-galactosidase, indicating the presence of incomplete species without any galactosyl residue. Because of their elution properties, it may be further concluded that they were also lacking, to a certain extent, outer GlcNAc residues. Since these glycans represented only minor constitu-
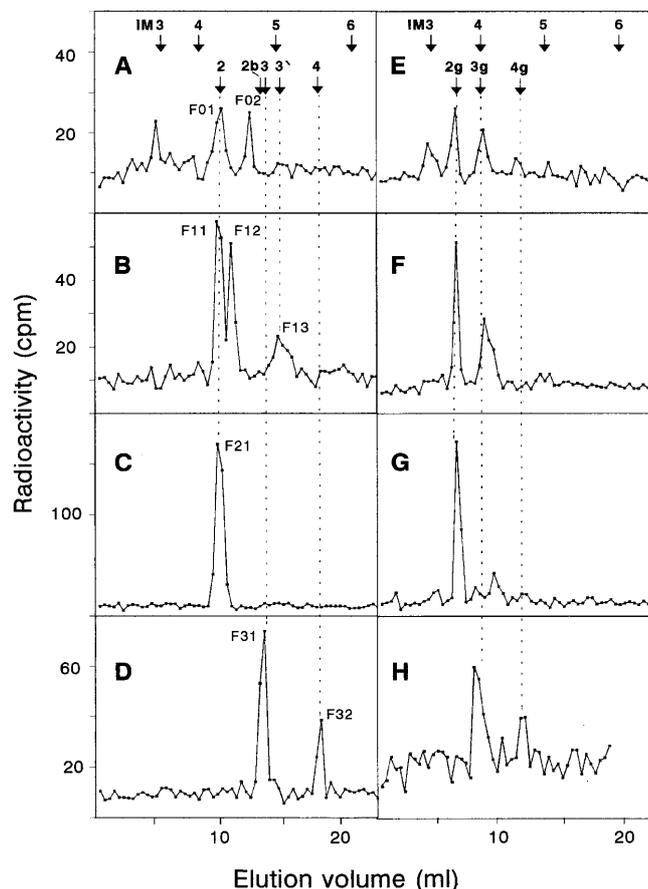
Fig. 5. Chromatographic profiles of neutral and desialylated complex-type glycans from human VE-cadherin. Oligosaccharide fractions obtained after preparative anion-exchange HPLC ((**A**), F0, (**B**), F1, (**C**), F2, (**D**), F3) were enzymatically desialylated and separated by HPAEC under the same conditions as in Figure 4. (**E-H**) The same fractions as in (**A–D**) after treatment with β-galactosidase from *D.pneumoniae*. Numbers (IM3-6) with arrows indicate the elution volumes of isomaltooligosaccharides with 3–6 glucose units; 2, 2b, 3, 3′, 4, elution volumes of fucosylated diantennary, bisected diantennary, 2,4-branched (3) and 2,6-branched (3′) isomers of triantennary and tetraantennary oligosaccharide standard alditols; 2g, 3g, 4g, elution volumes of the respective agalacto oligosaccharide standards (after degalactosylation, the two triantennary isomers and the bisected diantennary species coelute at 3g).

ents, they were not further analyzed. The structural conclusions, drawn from the analytical data described, are summarized in Table I.

Distribution of sialic acids and junctional cell adhesion molecules in endothelial cells in situ *and in culture*

Based on the glycosylation data described above, the localization of sialic acids and Ca^{2+}-dependent VE-cadherin was studied in endothelial cells of the human umbilical vein and arteries *in situ* as well as in culture (Figures 6, 7). As a control, Ca^{2+}-independent PECAM-1 was also localized (Figure 8). Digoxigenin labeled MAA- and SNA-lectins, used together or individually (MAA binds to α2,3-linked and SNA binds to α2,6-linked sialic acids), stained the intercellular junctions as well as surface proteins of endothelial cells *in situ* (Figure 6) and in culture (Figures 7, 8). Sialic acids, found at interendothelial junctions, largely colocalized with VE-cadherin in a not interrupted continuous pattern in endothelial cells of human umbilical vein and arteries *in situ*

Table I. Structures proposed for the major oligosaccharide fractions obtained from human VE-cadherin

Fraction	oligosaccharide structure	sialic acid substitution pattern		Molar ratio (mol/100 mol)
		α2-3	α2-6	
H3	Manα2Manα6⌐ 　　　　　Manα6⌐ Manα2Manα3⌐　　Manβ4GlcNAcOH Manα2Manα2Manα3⌐	-	-	9
H1	Manα6⌐ 　　Manα3⌐　　Manβ4GlcNAcOH Galβ4GlcNAcβ2Manα3⌐		1[a]	20
H2	Manα6⌐ 　　　　Manα6⌐ 　　Manα3⌐　　Manβ4GlcNAcOH Galβ4GlcNAcβ2Manα3⌐		1[a]	8
F01 F11 F21	Galβ4GlcNAcβ2Manα6⌐　　　　Fucα6⌐ 　　　　　Manβ4GlcNAcβ4GlcNAcOH Galβ4GlcNAcβ2Manα3⌐	- - 1(-)	- 1 1(2)[b]	40
F02 F12	⌐GlcNAcβ6/4⌐ 　　　　|GlcNAcβ2Manα6/3⌐　　　　Fucα6⌐ (Galß4)₁₋₂|　　　　　Manβ4GlcNAcβ4GlcNAcOH 　　　　⌊GlcNAcβ2Manα3/6⌐	- -	- 1	6
F13 F31	Galβ4GlcNAcβ6/4⌐ Galβ4GlcNAcβ2Manα6/3⌐　　　　Fucα6⌐ 　　　　　Manβ4GlcNAcβ4GlcNAcOH Galβ4GlcNAcβ2Manα3/6⌐	- 1(2)	1 2(1)[b]	9
F32	Galβ4GlcNAcβ6⌐ Galβ4GlcNAcβ2Manα6⌐　　　　Fucα6⌐ 　　　　　Manβ4GlcNAcβ4GlcNAcOH Galβ4GlcNAcβ2Manα3⌐ Galβ4GlcNAcβ4⌐	1(2)	2(1)[b]	1

Structures were deduced from the analytical results in accordance with the general rules of mammalian glycoprotein-N-glycan architecture (Vliegenthart and Montreuil, 1995; Sharon and Lis, 1997). The molar ratios were roughly estimated from the distribution of ^{3}H radioactivity incorporated into GlcNAc assuming a uniform labeling.
[a]Linkage position of sialic acid not assigned.
[b]Both sialylation variants possible.

(Figure 6) and in culture (Figure 7). In addition, at overlapping junctional areas of adjacent highly confluent endothelial cells a netlike distribution of VE-cadherin was observed (Figure 7A,B,D,E) that, again, largely colocalized with MAA/SNA-staining (Figure 7B1,D1,E1). This netlike structure has not been described before and represents a highly ordered VE-cadherin organization only observable in confluent endothelial cell cultures. Therefore, we termed it VE-cadherin superstructure. Double labeling of VE-cadherin and catenins also displayed a codistribution of this highly organized structure (not shown). PECAM-1, labeled with a monoclonal antibody, partially colocalized with MAA/SNA but to a lesser extent than VE-cadherin

(Figure 8C,D). This was further confirmed by serial optical z-sections using confocal laser microscopy. Whereas sialic acids and VE-cadherin were primarily localized within the apical area of intercellular junctions, PECAM-1 appears to reside predominantly at the basal side (data not shown).

Effect of extracellular Ca^{2+}-depletion on sialic acid distribution

To investigate whether junctional staining with MAA/SNA was mediated by sialic acids linked to Ca^{2+}-dependent molecules, Ca^{2+}-depletion experiments were performed. Incubation of cell monolayers with 3 mM EGTA for 30 min (leading to an

VE-cadherin   MAA/SNA
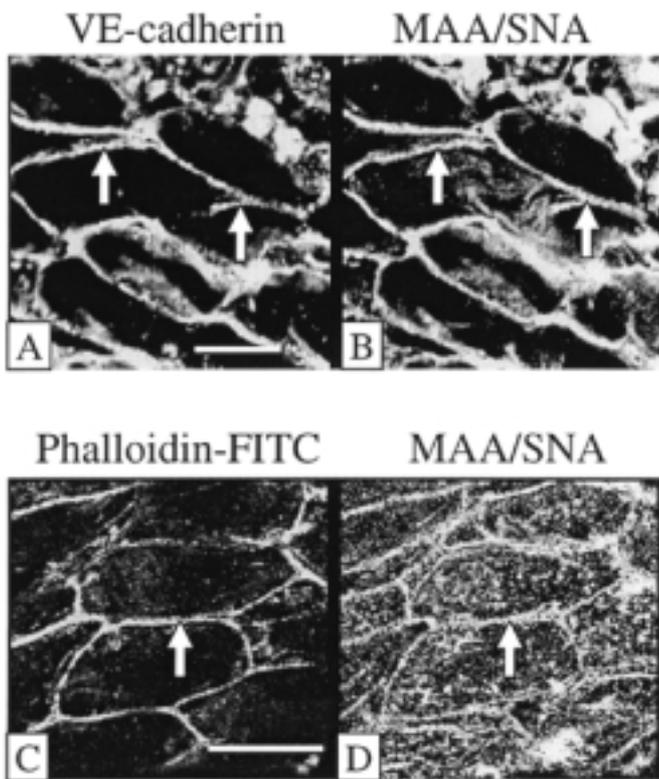
Phalloidin-FITC   MAA/SNA

Fig. 6. Colocalization of sialic acids, VE-cadherin and filamentous actin in endothelial cells of intact human umbilical veins *in situ* using confocal laser microscopy. Double staining of sialic acids (labeled by MAA/SNA) and VE-cadherin (labeled by a monoclonal antibody) (**A**, **B**) as well as sialic acids and actin filaments (stained by FITC-labeled phalloidin) (**C**, **D**). Both, VE-cadherin and the junctional located actin filaments display a codistribution with sialic acids (arrows in **A** and **B** as well as in **C** and **D** point to the very same cells). Scale bar, 20 μm.

extracellular Ca^{2+}-concentration of $<10^{-7}$ M) caused a complete absence of VE-cadherin and an almost complete disappearance of the MAA/SNA staining from interendothelial junctions (Figure 7C,C1) whereas PECAM-1 remained unchanged (Figure 8A,B). A very weak junctional staining of sialic acids, leftover after Ca^{2+}-depletion (Figure 7C1, 8A), might be caused by the remaining PECAM-1 and/or additional Ca^{2+}-independent components. After recalcification (1.8 mM of extracellular Ca^{2+}), VE-cadherin and MAA/SNA staining completely reappeared at the junctions within 30 min (Figure 7D,D1,E,E1). These findings are in line with previous observations showing a reappearance of VE-cadherin within 5 min after recalcification (Ayalon *et al.*, 1994; Dejana *et al.*, 1995; Schnittler *et al.*, 1997). They further indicate that sialic acids located at the interendothelial junctions are predominantly bound to Ca^{2+}-dependent molecules, presumably VE-cadherin. Preabsorption of MAA/SNA lectins with sialylated pig stomach type III mucin completely abolished the lectin staining in all experimental approaches.

Effect of sialic acid removal on VE-cadherin organization

Treatment of cultured endothelial cells with sialidase caused a complete loss of sialic acids from both the intercellular junctions and cell surface proteins without a loss of monolayer integrity. This was revealed by double-staining of sialidase treated cells with MAA/SNA-lectins and anti-PECAM-1 antibodies (Figure

8E,F). Under these conditions, VE-cadherin changed its continuous junctional distribution to a largely scattered morphology but was still localized at interendothelial junctions. Importantly, the VE-cadherin superstructure largely disappeared showing small protein clusters of various sizes indicating a loss of lateral adhesion between VE-cadherin molecules (Figure 7A,A1). Similar results were obtained when sialidase treatment was carried out in the presence of protease inhibitors ruling out that this observation might be due to contaminant proteolytic activities of the enzyme used (data not shown). In contrast, PECAM-1-immunostaining was completely maintained after sialidase treatment (Figure 8F) and demonstrated an intact endothelial cell monolayer in which the cells remained attached to each other.

Discussion

The structural characterization of the sugar chains of human VE-cadherin was based on chromatographic profiling by anion-exchange HPLC, HPAEC, and gel filtration in combination with exoglycosidase digestions. Since the separation systems used relied on different physicochemical parameters, comparison of the chromatographic data with those of oligosaccharide standards with known structures allowed a first structural assignment. Although anomeric configurations and linkage positions of the respective monosaccharide units were only unraveled in the case of sialic acid, galactosyl- and, in part, mannosyl residues, structures could be postulated on the basis of the general rules of mammalian glycoprotein-N-glycan architecture (Vliegenthart and Montreuil, 1995; Sharon and Lis, 1997). The results revealed that human VE-cadherin is substituted predominantly (~40% of total glycans) by sialylated diantennary complex-type glycans in addition to about 28% of sialylated hybrid-type species. Higher branched N-glycans, i.e., triantennary and, especially, tetraantennary chains as well as high mannose-type oligosaccharides were less abundant. Our data provided no evidence for the presence of oligosaccharides carrying "bisecting" GlcNAc as it has been shown by Nguyen and coworkers for bovine capillary endothelial cell carbohydrates (Nguyen *et al.*, 1992). Since the assignment of glycan structures is solely based on their chromatographic properties, however, the presence of small amounts of (eventually incomplete) bisected oligosaccharides cannot be completely ruled out. In conclusion, natural human VE-cadherin appears to be mainly decorated with carbohydrates of restricted branching pattern.

The high degree of sialylated oligosaccharide structures prompted us to visualize sialic acid residues at the surface of endothelial cells by lectin staining with MAA and SNA. Besides labeling of cell surface proteins, interendothelial junctions were strongly stained by these lectins which are specific for α2,3- and α2,6-linked sialic acids. The junctional appearance of MAA/SNA-labeling was largely restricted to VE-cadherin (observed by double labeling) whereas PECAM-1 appeared more extendent at the junctions both *in vivo* and in culture. In addition, although endothelial cells are thin reaching seldom more than 3 μm in height, the use of confocal laser microscopy allows a rough localization with a resolution of ~0.5 μm. By this technique, VE-cadherin immunofluorescence as well as MAA/SNA-labeling appeared predominantly at the apical pole of the junctions whereas PECAM-1 was primarily located at the basal pole. This is in line with previously published data obtained by immunoelectron microscopy demonstrating a basal localization of PECAM-1 and an apical localization of VE-cadherin within the interendothelial junctions (Ayalon *et al.*, 1994). Furthermore, Ca^{2+}-depletion
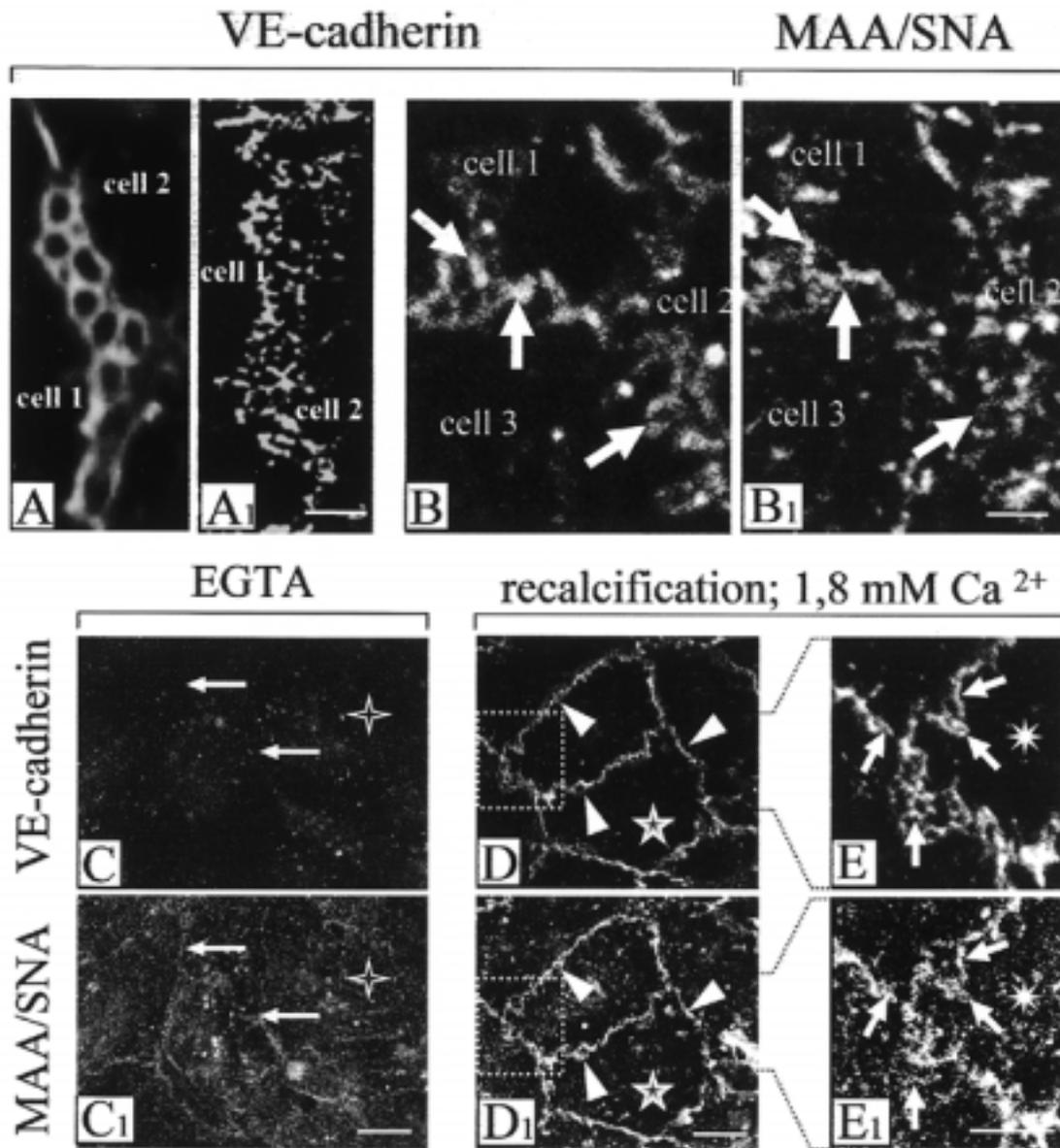
Fig. 7. VE-cadherin superstructure in highly confluent endothelial cultures and the effects of extracellular Ca^{2+}-depletion and sialic acid removal. Labeling of VE-cadherin by monoclonal antibody (**A**, **A1**) and double labeling of VE-cadherin by monoclonal antibody (**B–E**) and sialic acids by MAA/SNA (**B1**, **C1**, **D1**, **E1**) under control conditions (**A**, **B**, **B1**), after treatment with sialidase (**A1**), after treatment with 3 mM EGTA, $[Ca^{2+}] < 10^{-7}$ M, for 30 min (**C**, **C1**), and after recalcification, $[Ca^{2+}]$ 1.8 mM, for 30 min following EGTA treatment (**D**, **D1**, **E**, **E1**). Note, highly confluent cultures display a netlike VE-cadherin organization ("superstructure") at overlapping intercellular junctions between two adjacent cells (**A**) and at triangles between three or more cells (**B**). VE-cadherin is in general largely codistributed with sialic acids (**B**, **B1**). Removal of sialic acids caused a loss of the continuous VE-cadherin staining including a disappearance of the VE-cadherin superstructure (**A1**). Treatment with 3 mM EGTA completely abolished junctional labeling of VE-cadherin and largely the MAA/SNA staining (**C**, **C1**) that completely reappeared after recalcification including the VE-cadherin "superstructure" (**D**, **D1**, **E**, **E1**). Arrows and arrowheads indicate the same structures in corresponding double labeled figures. Identical stars mark the same cell. Scale bars: **A–B1**, 2.5 μm; **C–D1**, 20 μm; **E**, **E1**, 10 μm.

experiments showed that the junctional presence of sialic acids as well as the presence of VE-cadherin was reversibly dependent on extracellular Ca^{2+}-concentration whereas the junctional localization of PECAM-1 remained completely unchanged under all conditions. Additionally, it has been shown that VE-cadherin but not N-cadherin is clustered at the intercellular junctions (Salomon *et al.*, 1992; Navarro *et al.*, 1998). Hence, it may be assumed that junction located sialic acids might be primarily bound to VE-cadherin, which is in agreement with the carbohydrate analyses of purified VE-cadherin. The remaining weak MAA/

SNA staining after Ca^{2+}-depletion might be related to Ca^{2+}-independent molecules such as PECAM-1.

In highly confluent cultures of human umbilical vein and artery endothelial cells, VE-cadherin appeared in an undisturbed continuous band along the interendothelial junctions. At overlapping endothelial cell junctions, a netlike VE-cadherin organization was visualized. This network can be considered as extended VE-cadherin clusters and is assumed to considerably increase the interendothelial adhesion properties. It has been shown by crystal structural analysis that cadherins are obviously organized as
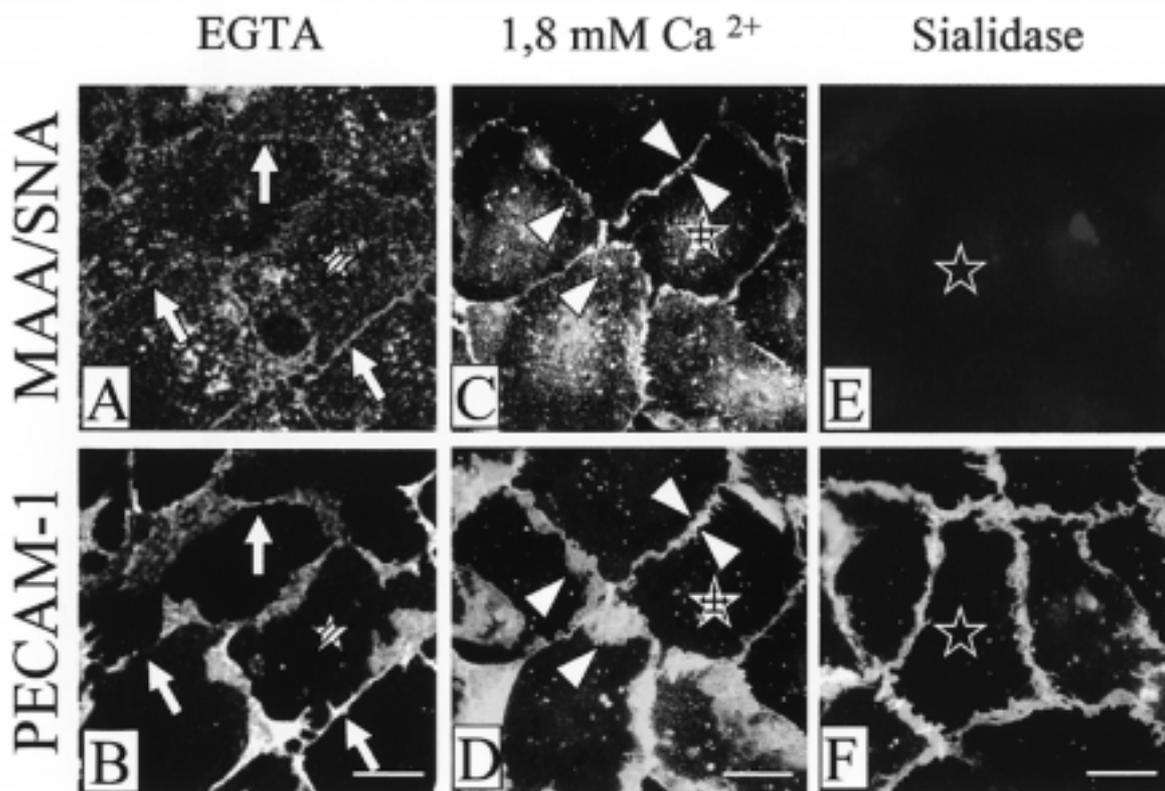
Fig. 8. Localization of sialic acids and PECAM-1 after extracellular Ca^{2+}-depletion, recalcification, and sialidase treatment in cultured human umbilical vein endothelial cells. Double labeling of sialic acids by MAA/SNA (**A, C, E**) and PECAM-1 by monoclonal antibody (**B, D, F**) after treatment with EGTA, $[Ca^{2+}]$ $<10^{-7}$ M, for 30 min (**A, B**), recalcification, $[Ca^{2+}]$ = 1.8 mM, for 30 min after EGTA treatment (**C, D**), or sialidase treatment for 60 min (**E, F**). MAA/SNA lectins are partially codistributed with PECAM-1 but there are also areas that are stained by PECAM-1 but not by MAA/SNA (**C, D**, arrowheads). In contrast to VE-cadherin (compare Figure 7) PECAM-1 remained completely unchanged after EGTA (**B**) or sialidase treatment (**F**). Note, MAA/SNA labeling was almost lost in the presence of EGTA. Only a weak MAA/SNA label was observed after EGTA treatment (**A**, arrows). A complete loss of MAA/SNA-labeling was registered after sialidase treatment (**E**). Stars indicate same cells. Scale bars, 20 μm.

"parallel strand dimers" that interact with "parallel strand dimers" of opposite cells (adhesion dimers) forming a zipper-like structure (Shapiro *et al.*, 1995a,b). The formation of such a superstructure possibly requires lateral association of the assumed cadherin strand dimers that might be influenced by carbohydrate residues. The discussion on the contribution of glycan chains to VE-cadherin function, however, is still contradictory. Yoshimura and co-workers (Yoshimura *et al.*, 1995, 1996) provided evidence for a functional role of E-cadherin glycosylation in that murine melanoma B16-hm cells transfected with the (β1–4)-*N*-acetylglucosaminyltransferase (GnT-III) cDNA showed a higher expression of E-cadherin at cell–cell contacts than control cells. Since the presence of bisecting GlcNAc residues is known to block further branching of glycoprotein-N-glycans (Schachter, 1986, 1995; Fujii *et al.*, 1990), respective glycans can be assumed to remain predominantly in the diantennary state. Therefore, the authors conclude that the reduced branching pattern of E-cadherin glycans, induced by ectopically expressed GnT-III, might be responsible for an elevated expression at the cell–cell border. This observation is in good agreement with our results revealing mainly diantennary and hybrid-type glycans on natural VE-cadherin. On the other hand, it has been observed that E-cadherin containing F9 cells still aggregate after tunicamycin treatment suggesting a glycan independent E-cadherin adhesive function (Shirayoshi *et al.*,

1986). Our data show that sialidase treatment of living endothelial cells caused a significant change in VE-cadherin cellular organization. Under these conditions, VE-cadherin still appeared at interendothelial junctions but displayed a scattered immunofluorescence pattern including the disappearance of its superstructure. In contrast, PECAM-1 underwent no morphological changes. Thus, the results described may at least suggest an involvement of sialic acid residues in the structural organization of VE-cadherin. The question, as to whether this finding depends, in fact, on sialic acid residues linked to VE-cadherin glycans, remains open since we cannot exclude that removal of sialic acids from the cell surface may cause indirect effects on VE-cadherin organization, as well.

In conclusion, our results demonstrate that (1) VE-cadherin is substituted with oligosaccharide side chains of reduced branching pattern which are highly sialylated, (2) sialic acids present at interendothelial junctions are predominantly bound to Ca^{2+}-dependent molecules, (3) sialic acids are largely codistributed with VE-cadherin molecules, and (4) VE-cadherin superstructural- but not PECAM-1-organization is lost after sialidase treatment. From the above results, one might speculate that VE-cadherin glycan chains represent the backbone for the presentation of sialic acids which might be involved in Ca^{2+}-binding and, thus, in the maintenance of the rod-like VE-cadherin structure and its superstructural organization. Further studies are required, however, to

definitely prove the influence of carbohydrate substituents on VE-cadherin function.

Materials and methods

Cell culture

Human umbilical vein endothelial cells (HUVEC) were harvested and cultured as previously described (Schnittler *et al.*, 1993b). HUVEC were cultured in Medium 199 (Gibco, Eggenstein, Germany) supplemented with 20% pooled human serum obtained from healthy donors of the local blood bank, 50 μg/ml streptomycinsulfate and 50 U/ml penicillin G (Sigma, Deisenhofen, Germany). HUVEC were seeded on glass coverslips coated with cross-linked gelatin as described (Schnittler *et al.*, 1993a). Cells from the first and second passages were used for the experiments.

Antibodies, lectins, and immunofluorescence staining

Monoclonal mouse antibody to VE-cadherin were purchased from Biermann GmbH (Bad Nauheim, Germany), polyclonal rabbit antibody to pan cadherin (known to cross-react with a cytoplasmic carboxyterminal domain of classical cadherins), and FITC-labeled and TRITC-labeled phalloidin was from Sigma (Deisenhofen, Germany), and to PECAM-1 from R&D Systems (Wiesbaden, Germany). Digoxigenin-labeled MAA (from *Maakia amurensis*), SNA (from *Sambucus nigra*), and TRITC-labeled sheep anti-digoxigenin antibody were obtained from Boehringer (Mannheim, Germany). Fluorescein isothiocyanate- (FITC), tetramethyl isothiocyanate- (TRITC), and cyanine 3- (Cy3) labeled secondary antibodies were from Dianova (Hamburg). For lectin, antibody, and phalloidin labeling, fresh human umbilical cord vessels were canulated and perfused with Medium 199 to remove blood components and subsequently fixed with 2% formaldehyde in phosphate-buffered saline (PBS containing 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4; pH 7.4). Arteries and veins were cut out and further processed for lectin and antibody labeling as described below. Cultures of human umbilical vein and artery endothelial cells were washed with serum free Medium 199 and subsequently fixed in 2% formaldehyde dissolved in PBS. Pieces of umbilical veins, arteries as well as cultured cells, were washed several times with PBS and permeabilized with 0.1% Triton X-100 (Sigma). Samples were incubated with 1μg/ml digoxigenin labeled MAA/SNA or phalloidin for 10 min at room temperature or with mouse monoclonal antibodies directed to VE-cadherin (diluted 1:50 with PBS) or PECAM-1 (diluted 1:50 with PBS) overnight (4°C). Samples were then washed with PBS and incubated for 60 min with FITC- or Cy3-labeled goat anti-mouse IgG or TRITC-labeled sheep anti digoxigenin Fab fragments. For double-labeling of lectins (MAA and SNA) and specific antibodies directed to VE-cadherin as well as PECAM-1, cells were fixed with 2% formaldehyde and incubated contemporary with MAA/SNA and the appropriate primary and secondary antibodies as described above. After several washes with PBS (15 min), the coverslips were mounted on glass slides covered with 60% glycerol and 1.5% n-propylgallate as an antifading substance. To verify that lectin staining at interendothelial junctions was specific for sialic acids, type III mucin from porcine stomach (Sigma, Deisenhofen, Germany) was used for preabsorption of MAA and SNA lectins.

Ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA) treatment

EGTA treatment was performed as described elsewhere (Schnittler *et al.*, 1997). Briefly, cells were treated with 3 mM EGTA dissolved in Medium 199 supplemented with 1% of human albumin essentially free of fatty acids and globulins. The free $[Ca^{2+}]$ was calculated using a Ca^{2+} calculation program (Föhr *et al.*, 1993) and was $<10^{-7}$ M in all media. The pH was adjusted to 7.4 in EGTA stock solution (300 mM) with 5 M NaOH.

Sialidase treatment of endothelial monolayers

Sialidase treatment was performed according to a protocol described previously (Krempl *et al.*, 1997). Briefly, cell monolayers were three times washed with Medium 199 containing 1% of immunoglobulin- and fatty acid-free human albumin (Sigma, Deisenhofen, Germany) or 10% of serum and subsequently incubated with highly purified sialidase from *Clostridium perfringens* (Sigma, Deisenhofen, Germany) at 200 mU/ml for 60 min at 37°C, and subsequent experiments were performed in the presence of sialidase. Sialidase treatment was also performed in the presence of protease inhibitors (aprotinin, pepstatin, and leupeptin, 20 μg/ml each). Under all experimental conditions (presence or absence of serum or inhibitors) sialidase treatment caused a complete loss of sialic acids from the cells.

Metabolic labeling

Carbohydrate substituents were labeled according to a protocol described previously (Geyer *et al.*, 1992). Briefly, HUVEC of passages zero and one were cultured in 75 cm^2 culture flasks (10×) to confluence. Prior to metabolic labeling, monolayers were washed in glucose-deficient Medium 199 (Biochrom, Berlin, Germany), supplemented with 1.8 g/l fructose, 10% pooled human serum (obtained from healthy donors of the local blood bank and dialyzed against glucose-free Medium 199), 50 μg/ml streptomycinsulfate, and 50 U/ml penicillin G, and further cultivated for 2 h. Cells were washed again and subsequently labeled with 20 μCi/ml D-[6-^{3}H]glucosamine (Amersham Buchler, Braunschweig, Germany). After a labeling period of 20 h in glucose-deficient medium, cultures were washed with PBS (3 × 20 ml, 4°C) followed by a brief rinse with Triton X-100–free extraction buffer (see below).

Cell extraction

All steps were performed at 4°C, and buffers used contained 1 mM phenylmethylsulfonyl fluoride, 25 μg/ml leupeptin, 25 μg/ml aprotinin, and 25 μg/ml pepstatin (Sigma). For cell extraction, 500 μl of extraction buffer (20 mM Tris/HCl, 150 mM NaCl, 1 mM $CaCl_2$, 0.04% sodium azide, 1% Triton X-100, pH 8) were used for one 75 cm^2 culture flask. After 15 min of incubation, cells were scraped off the substratum using a rubber policeman. Subsequently, samples were centrifuged for 5 min at $14,000 \times g$.

Enzymatic digestion of total cell extracts

Carbohydrate substituents of VE-cadherin were investigated by Western blotting before and after PNGase F and endo H digestion. HUVEC were scraped from culture flasks in 1 ml Medium 199 containing 20% pooled human serum, 25 μg/ml leupeptin, 25 μg/ml aprotinin, and 25 μg/ml pepstatin at 4°C. After two washes with serum-free Medium 199 cells were extracted with

buffer (0.1% SDS, 0.5% octylglycoside, 0.5% β-mercaptoethanol), sonicated for 30 s, boiled for 5 min, and then centrifuged for 5 min at 8000 × *g*. For digestion with PNGase F from *Flavobacterium meningosepticum*, aliquots of the supernatants were adjusted to 50 mM sodium acetate, 5 mM EDTA, 0.04% sodium azide (pH 7), and 10 U/ml PNGase F using stock solutions. Samples were incubated at 37°C for 48 h. After 24 h, PNGase F (10 U/ml) was added again. Control experiments were performed with water instead of enzyme. Treatment with endo H from *Streptomyces griseus* was performed in the same way using 100 mU/ml endo H at pH 5.5.

Purification of VE-cadherin

VE-cadherin was isolated from the supernatants of cell extracts by immunoprecipitation using mouse monoclonal VE-cadherin antibody (20 μg) coupled to Protein G Sepharose beads (75 μl; Pharmacia, Upsala, Sweden). After incubation over night under continuous rotation, the Sepharose beads were washed three times with 2 ml washing buffer 400 (50 mM Tris/HCl; 400 mM NaCl, 1 mM CaCl$_2$, 0.04% sodium azide, 0.05% Triton X-100, 1 mg/ml ovalbumin, pH 8.4) and one time with washing buffer 150 (50 mM Tris/HCl, 150 mM NaCl, 1 mM CaCl$_2$, 0.04% sodium azide, 0.05% Triton X-100, 1 mg/ml ovalbumin, pH 8.4). Samples were dissolved in sample buffer, boiled for 5 min, and applied to preparative SDS-polyacrylamide gels (10% polyacrylamide) (Schnittler *et al.*, 1990). After electrophoresis, SDS gels were dried and radiolabeled protein bands were detected by fluorography. Gel segments containing VE-cadherin were excised. Western blots were exactly performed as described previously (Schnittler *et al.*, 1990).

In-gel proteolytic digestion

The excised gel pieces from three SDS gels (22 slots in total) were cut into pieces of about 1 mm^2, extensively washed with twice-distilled water, and completely dried in a SpeedVac concentrator. Dried gel pieces were suspended in 50 μl of acetonitrile and 500 μl of 50 mM Tris/HCl, pH 8.0, containing 1.2 μg of endoproteinase Asp-N from *Pseudomonas fragi* mutant (Boehringer, Mannheim, Germany). After overnight incubation at 35°C, again 20 μl of acetonitrile and 200 μl of buffer with enzyme (1 μg) were added and incubated at room temperature for further 24 h. Glycopeptides were recovered from the gel pieces by removing the incubation buffer and washing gel pieces five times with 10 mM Tris/HCl buffer and sonication. Combined supernatants were lyophilized and desalted by gel filtration.

Isolation of oligosaccharides

N-Linked glycans were released from glycopeptides by sequential treatment with endo H from *Streptomyces plicatus* and PNGase F from *Flavobacterium meningosepticum* (both from Boehringer), separated from residual (glyco)peptides, reduced, and desalted as described previously (Strube *et al.*, 1988; Geyer *et al.*, 1992; Geyer and Geyer, 1993).

Chromatographic procedures

Desalting of glycopeptides and free oligosaccharides by Bio-Gel P-2 chromatography or by ion-exchange chromatography using a mixed-bed resin (Amberlite AG-MB3; Serva, Heidelberg, Germany), separation of oligosaccharides from (glyco)peptides by RP-HPLC at pH 6.0, fractionation of glycans by anion-

exchange HPLC using a Mikropak AX-10 column (Varian, Walnut Creek, CA) and by HPAEC using a CarboPak PA-100 column (Dionex, Sunnyvale, CA) as well as gel filtration on a Bio-Gel P-4 column were performed as described previously (Strube *et al.*, 1988; Geyer and Geyer, 1993; Liedtke *et al.*, 1994). Radiolabeled monosaccharide components were identified by HPAEC as detailed previously (Geyer *et al.*, 1992).

Degradation of oligosaccharides

Glycans were enzymatically digested with sialidases from *Vibrio cholerae* (Behringwerke, Marburg, Germany) or Newcastle disease virus (Oxford GlycoSystems, Abingdon, UK), β-galactosidase from *Diplococcus pneumoniae*, and α-mannosidase from jack beans (Boehringer) using the same conditions as described previously (Geyer *et al.*, 1992; Liedtke *et al.*, 1997). For mild acid hydrolysis, glycans were hydrolyzed in 500 μl of 1 N trifluoroacetic acid for 30 min at 80°C and dried in a SpeedVac concentrator. Residual acid was removed by addition of 2 × 1 ml of methanol and evaporation under vacuum.

Abbreviations

Cy3, cyanine 3; EGTA, ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; endo H, endo-β-*N*-acetylglucosaminidase H from *Streptomyces plicatus*; FITC, fluorescein isothiocyanate; Fuc, fucose; Gal, galactose; GlcNAc, N-acetylglucosamine; GlcNAcOH, *N*-acetylglucosaminitol; HUVEC, human umbilical vein endothelial cells; MAA, *Maakia amurensis* agglutinin; Man, mannose; HPAEC, high-pH anion-exchange chromatography; PECAM-1, platelet endothelial cell adhesion molecule; PNGase F, peptide-*N*^{4}-(*N*-acetyl-β-glucosaminyl)asparagine amidase F from *Flavobacterium meningosepticum*; RP-HPLC, reverse-phase HPLC; SNA, *Sambucus nigra* agglutinin; TRITC, tetramethylrhodamine isothiocyanate; VE-cadherin, vascular endothelial cadherin.

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