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ARTICLE

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Plasmalemmal concentration and affinity of mouse vascular endothelial cadherin, VE-cadherin

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Abstract Ca^{2+}-dependent adhesion molecules, cadherins, are critically involved in the barrier formation of epithelial layers. Adhesive strength depends on both the plasmalemmal concentration and adhesive affinity (affinity for *trans* interaction) of cadherins. In the present study we used recombinant vascular endothelial cadherin, VE-cadherin, as a reference to quantify the surface concentration of VE-cadherin in mouse microvascular endothelial cells by linear interpolation and regression analysis of immunosignals obtained with cell lysates dotted on nitrocellulose membranes. The affinity of *trans* interaction was determined by a novel mobility shift assay, in which soluble dimeric VE-cadherin ectodomains pass through a VE-cadherin affinity column. By these approaches we determined the trypsin-sensitive surface concentration of VE-cadherin to be 5×10^3 dimers/μm^2 cell surface and the dissociation constant K_D to be about 0.8×10^{-4} M. The low affinity of *trans* interaction in combination with high plasmalemmal concentration of VE-cadherins fulfils theoretical predictions for regulation of adhesion by a transmembrane cooperative linkage mechanism, in which the degree of lateral mobility (translational entropy) of cadherins in the plasma membrane determines the number of adhesive bonds and, hence, the strength of intercellular adhesion.

Keywords Chromatography · Endothelium · Quantitative blot · Mobility shift · Cadherin 5

Introduction

Selective adhesion between cells is mediated by different families of adhesion molecules (Hynes and Zhao 2000), among which the Ca^{2+}-dependent adhesion molecules, cadherins, are critically involved in a variety of important physiological and pathological processes (Gumbiner 2000; Angst et al. 2001). Cadherins are single-span type I membrane proteins that require millimolar concentrations of free extracellular Ca^{2+} for *trans* interaction of their ectodomains with cadherins of adjoining cells. The ectodomain consists of five cadherin-specific subdomains (EC1–EC5), which undergo *cis* homodimerization in physiological Ca^{2+} and is assumed to contain one or two binding sites for *trans* interactions (Shapiro et al. 1995; Yap et al. 1998; Koch et al. 1999; Takeda et al. 1999). The short cytodomain of classical cadherins contains binding sites of cadherin-specific adaptor molecules, catenins, which serve to link cadherins to actin filaments. This cytoskeletal anchorage appears to be important for the strengthening of adhesion (Kemler and Ozawa 1989; Yap et al. 1997; Angst et al. 2001).

Adhesive contacts must be able to rapidly assemble and dissociate to allow dynamic cellular behaviour to occur, such as morphogenetic cell rearrangements, several steps of tumour metastasis (Christofori and Semb 1999), and opening and closing of junctions between vascular endothelial cells to control paracellular passage of macromolecules and leucocytes (Vestweber 2000). Formation and dissociation of adhesive contacts implies mechanisms that are able to modulate the adhesive strength of cadherin-based junctions between cells. It is presently not well understood how adhesion between cadherins can be regulated by intracellular signalling events. A widely favoured model for regulation of cadherin-mediated adhesion is based on the observation that these adhesive contacts require linkage of cadherins to the actin filament system (Angres et al. 1996; Yap et al. 1997; Angst et al. 2001; Vasioukhin and Fuchs 2001; Baumgartner and Drenckhahn 2002). Any signalling event that interferes with catenin-based attachment of cadherins to the actin filament system is believed to play a critical role in regulation of adhesive strength. However, we have recently ruled out transmembrane affinity changes of vascular endothelial cadherin

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(VE-cadherin) by cytoskeletal tethering (Baumgartner et al., submitted for publication).

In a theoretical evaluation of this aspect we proposed a thermodynamic model, in which damping of the lateral mobility (translational entropy) of cadherins in the plane of the lipid bilayer by linkage to the cytoskeleton will facilitate rapid rebinding of cadherins after dissociation, thereby effectively increasing the number of cadherin bonds between the contacting membrane surfaces (Baumgartner and Drenckhahn 2002). However, such a transmembrane cooperative linkage mechanism would only be effective at rather high dissociation constants in the millimolar range and, accordingly, depends strongly on the overall concentration of cadherins in the plane of the membrane.

By single molecule atomic force measurements (AFM), we have recently determined the apparent k_{off} and k_{on} of recombinant VE-cadherin, in which the complete ectodomain of mouse VE-cadherin was fused to the Fc portion of human IgG (VE-cadherin-Fc) (Baumgartner et al. 2000a, 2000b). The apparent low affinity of VE-cadherin-Fc determined by AFM (millimolar range) demanded proof by other methods. Plasmon resonance measurements did not provide unequivocal data, probably because of the extremely low affinity. In the present study, we have developed an affinity column assay to determine K_D by mobility shift. This assay provides a promising approach for the determination of affinities even at millimolar K_D values. The results obtained by this approach confirm our estimation of K_D by AFM studies. The low affinity of the VE-cadherin *trans* interaction appears to be partly compensated by a rather high level of surface expression of VE-cadherin, which we determined in this study to be in the range of 5×10^3 *cis* dimers/μm^2 cell surface.

Materials and methods

VE-cadherin-Fc

A VE-cadherin-Fc fusion protein was generated as described recently (Moll and Vestweber 1999; Baumgartner et al. 2000a). Briefly, a DNA fragment coding for the complete extracellular part of mouse VE-cadherin, including the membrane proximal glutamine, was placed in front of a cDNA fragment coding for the Fc part of human IgG1, including the hinge region and Ig domains C_H2 and C_H3. CHO cells were stably transfected and the secreted VE-cadherin-Fc chimera were purified from the culture supernatants by affinity chromatography using protein A agarose. The purified protein was characterized as described.

For quantitative dot blot analysis, VE-cadherin-Fc was split into monomeric strands by reduction of disulfide bonds between the Fc portion of the fusion protein. For this purpose, 100 μg of lyophilized VE-cadherin-Fc was dissolved in 400 μL cleaving solution (50 mM DTT, 500 mM Tris-HCl, pH 8.0) and incubated for 60 min at room temperature (RT). Free SH groups were blocked by addition of 0.2 mL of 0.3 M iodoacetamide. Solute exchange against phosphate-buffered saline (PBS, pH 7.4) was performed by centrifugation at 14,000×*g* (20 min) using Amicon centrifugation filters (Ultrafree-MC, 30,000 MG, Milipore, Bradford, Mass., USA). Western blotting confirmed formation of monomers, which migrated as single bands at $\sim90K_D$, which corresponds precisely to one half of the calculated molecular weight of the VE-cadherin-Fc fusion protein (180,000; Baumgartner et al. 2000a).

Cell cultures

Immortalized mouse microvascular endothelial cell line (MyEnd) was obtained from mouse myocardium by transfection with polyoma middle T antigen. MyEnd cells have been characterized in detail previously (Adamson et al. 2002). Cells were grown in Dulbeccos modified Eagles medium (DMEM; Life technologies, Karlsruhe, Germany), supplemented with 50 U/mL penicillin-G, 50 μg streptomycin, and 10% fetal calf serum (Biochrom, Berlin, Germany) in a humidified atmosphere (95% air/5% CO_2) at 37 °C. The cultures were used for experiments at an age of 3–5 days (fully confluent monolayers). Madin-Darby canine kidney (MDCK) cells, clone 11 (Gekle et al. 1994), were cultured under identical conditions and used as negative controls.

Quantitative dot blot analysis

Confluent monolayers were scraped by a rubber policeman from a 25 cm^2 culture dish and suspended in 300 μL of Hanks balanced salt solution (HBSS) containing 0.1% sodium dodecyl sulfate (SDS) and a protease inhibitor cocktail consisting of leupeptin, aprotinin, and pepstatin (10 μg/mL each). The suspension was sonified for 1 min with a Bradson 250 sonifier (Heinemann, Ultraschalltechnik, Schwäbisch Gmünd, Germany) at maximal setting and the protein content was determined by the Bradford method (Bradford 1976). The number of cells covering 25 cm^2 was determined by a CASY 1 cell counter (Casy, Schärfe Systems, Reutlingen, Germany).

For standard dot blots, 1 μg, 5 μg, and 10 μg protein of endothelial and MDCK cell lysates were adsorbed to individual dots onto Hybond nitrocellulose membranes (Amersham, Braunschweig, Germany). As reference for quantification, defined amounts of monomeric VE-cadherin-Fc were dotted onto the same sheet of nitrocellulose.

To rule out masking of VE-cadherin by other endothelial proteins or lipids present in the lysate, additional control experiments were performed in which each dot was loaded with VE-cadherin-Fc plus 5 μg of endothelial proteins (lysate). After blocking of excess protein binding sites on nitrocellulose with 5% low fat milk in PBS (3 h, RT), nitrocellulose sheets were incubated overnight with monoclonal antibody 11D4.1 (Gotsch et al. 1997), which is directed against the extracellular domain of VE-cadherin (hybridoma supernatant kindly provided by D. Vestweber, Münster, Germany). As a secondary antibody, horseradish peroxidase-labelled goat anti-rat IgG (Jackson Immuno Res. Lab., West Grove, Pa., USA) was used at a dilution of 1:2000 in PBS. Bound IgGs were visualized by enhanced chemiluminescence (ECL, Amersham).

Trypsin treatment of endothelial cells

MyEnd cells, grown on 25 cm^2 culture dishes, were incubated at 37 °C with 2 mL of 0.05% (w/v) trypsin dissolved either in HBSS (containing 2 mM Ca^{2+}) or in PBS containing 2 mM EGTA for 7 min. After removal of the trpysin-containing supernatant, monolayers were washed three times with PBS containing a mixture of protease inhibitors (leupeptin, aprotinin, pepstatin; 10 μg/mL each, Sigma). Afterwards, cells were scraped from the culture dish and dissolved in 300 μL pre-heated Laemmli buffer (60 °C, 3 min; Laemmli 1979) containing the same protease inhibitors. The lysates were sonified and subjected to SDS polyacrylamide (10%) gel electrophoresis (SDS-PAGE). For immunoblotting, proteins were transferred in Kyhse-Andersen transfer buffer (Kyhse-Andersen 1984) to Hybond nitrocellulose membranes and processed for immunodetection of the VE-cadherin ectodomain using 11D4.1 antibody and the ECL technique (see preceding paragraph).

Chromatography

Affinity chromatography

CNBr-activated sepharose (100 mg; Sigma, Taufkirchen, Germany) was allowed to swell for 45 min at 4 °C in 1 mM HCl (10 mL). The swollen sepharose (~1 mL) was transferred to a column (diameter 5 mm) and washed with 100 mL of 1 mM HCl, followed by 3 mL of distilled water (H_2O). The column was equilibrated with 1 mL of coupling buffer (100 mM $NaHCO_3$, 500 mM NaCl, pH 8.4) and then loaded with 1 mL of coupling buffer containing a mixture of 0.8 mg/mL VE-cadherin-Fc and 0.8 mg/mL bovine serum albumin (BSA) and allowed to react for 2 h at RT under slow overhead rotation. Afterwards the column was washed once with 3 mL coupling buffer, followed by a wash with 300 mL blocking buffer (200 mM glycine, pH 8.0) and then subjected to incubation for 3 h at RT in blocking buffer. After three washes with 3 mL acetate buffer (100 mM acetate, 500 mM NaCl, pH 4.5), the column was washed and equilibrated with either 10 mL of HBSS containing 2 mM Ca^{2+} or PBS.

For determination of the mobility shift, typically 25 µL (1.13 mg/mL) of VE-cadherin-Fc in HBSS (without or with 2 mM EGTA) was loaded onto the column and allowed to flow through with a velocity of ~0.14 mL/min. Fractions of either 60, 80, or 120 µL were collected and subjected to dot blot analysis and determination of the protein amount (see above).

Control column

For control experiments, CNBr-sepharose was coupled with 1.6 mg BSA in the absence of VE-cadherin-Fc.

Gel filtration chromatography

To determine whether VE-cadherin-Fc might oligomerize (aggregate) during passage through column, 25 µL VE-cadherin-Fc (containing 1.3 mg/mL in HBSS without or with 2 mM EGTA) was run through a 500 µL sephacryl S300 column with the same dimensions. Fractions of 80 µL were used for further analysis of protein content.

Data analysis for affinity chromatography

Single-molecule AFM experiments indicated low-affinity *trans* interaction of VE-cadherin-Fc in the millimolar range. Therefore, the fraction of VE-cadherin-Fc of the soluble phase that *trans* interacts with VE-cadherin-Fc covalently coupled to the column material (solid phase) was expected to be negligible in comparison to the total concentration of VE-cadherin-Fc in solution. Under these conditions, the concentration profile of protein in the soluble phase will behave according to the partial differential equation:

$$\frac{\partial c}{\partial t} = \frac{D}{c_i K_D}\frac{\partial^2 c}{\partial x^2} - \frac{v}{c_i K_D}\frac{\partial c}{\partial x} \quad (1)$$

with c denoting the concentration of protein in the soluble phase, c_i the concentration of protein in the solid phase, K_D the dissociation constant of monovalent *trans* interaction between soluble and solid phase proteins, and v denoting the liquid flow velocity. Assuming the initial concentration to be a delta function containing a total concentration of protein (c_{tot}) at zero x-position ($x=0$), the solution follows to be:

$$c = \frac{c_{tot}\sqrt{c_i K_D}}{\sqrt{2\pi D t}} \exp\left[-\frac{\left(x - vt(c_i K_D)^{-1}\right)}{2Dt} c_i K_D\right] \quad (2)$$

For mathematical simplicity, the cumulative protein concentration of the obtained fractions normalized to the total protein concentration was fitted to the integral over time of the function given in Eq. (2). The fitting was performed using weighted least squares. Because of the natural limits of the normalized cumulative protein concentration at 0 and 1, the least square deviations x were weighted by $x(1-x)$.

Results

The total concentration of VE-cadherin in microvascular endothelial cells was determined by densitometry of dot blots of lysates of endothelial cells scraped from the culture dish. Grey values of ECL signals were calibrated by separate dot blots on the same nitrocellulose sheets loaded with known amounts of monomeric VE-cadherin-Fc (assay A). MDCK cells served as negative controls (no immunosignal with VE-cadherin antibody). Figure 1 shows a typical example of dot blots of a dilution series of lysates of two different endothelial monolayers (a, b) and monomeric VE-cadherin-Fc (c). Linear interpolation of grey values allowed determination of the VE-cadherin content of ~0.064 µg VE-cadherin per 5 µg cellular protein (12.8 ng/µg). To rule out the possibility of masking effects of cellular components of cell lysates, increasing amounts of monomeric VE-cadherin-Fc were added to the lysates (assay B). Figure 2 shows typical examples of three experiments which allow determination of VE-cadherin by exponential regression of grey values. By this approach, VE-cadherin content of 5 µg total protein of cell lysate (m_{sample}) corresponds to 0.076 µg monomeric VE-cadherin-Fc ($m_{VE\text{-}Fc}$) (15.2 ng/µg). The molecular weight of monomeric VE-cadherin-Fc is $MW_{VE\text{-}Fc} \approx 90{,}000$ and the total amount of protein obtained from MyEnd monolayers covering 25 cm^2 culture dish was $m_{tot} \approx 680$ µg. Approximating the total dorsal, ventral, and lateral plasma membrane of 25 cm^2 of endothelial monolayer (~10^6 cells, average depth of intercellular clefts ~3 µm) to be $A \approx 55$ cm^2 and assuming that all cadherin molecules (14.2 ± 1.7 ng/µg, average ± SD from nine experiments of assay A and three experiments of assay B) are inserted into the plasma membrane, it is possible to calculate the surface concentration of VE-cadherin ($c_{surf} = N_A \times m_{VE\text{-}Fc} \times m_{tot} / [m_{sample} \times MW_{VE\text{-}Fc} \times A]$) to be approximately 10^4 cadherin

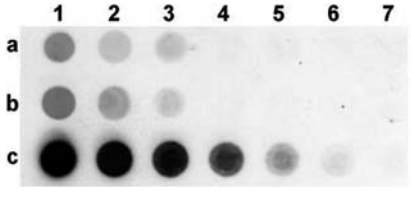

Fig. 1 Dot blot assay of two endothelial cell lysates (*a*, *b*) and of purified recombinant monomeric VE-cadherin-Fc (*c*) dotted on the same nitrocellulose sheet and probed simultaneously with mAb 11D4.1. Protein amounts of cell lysates loaded per dot were 10 µg (dot 1), 5 µg (dot 2), and 1 µg (dot 3). Recombinant VE-cadherin-Fc loaded per dot was 5 µg (dot 1), 5/3 µg (dot 2), 5/9 µg (dot 3), 5/27 µg (dot 4), 5/81 µg (dot 5), and 5/243 µg (dot 6)

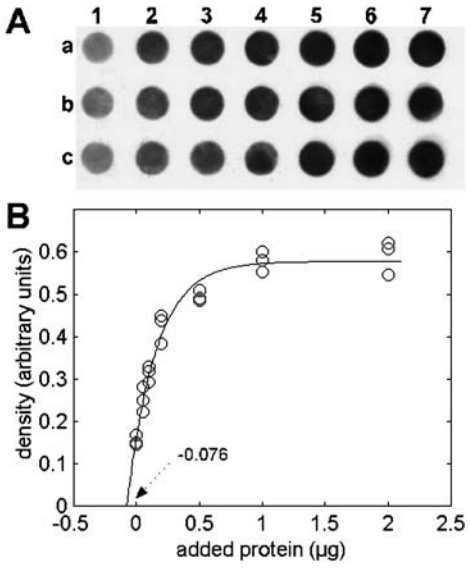

Fig. 2 **A** Dot blot assays of cell lysates (5 µg each) of three different cultures (*a–c*) to which increasing amounts of monomeric VE-cadherin-Fc was added: 0 µg (dot 1), 0.05 µg (dot 2), 0.1 µg (dot 3), 0.2 µg (dot 4), 0.5 µg (dot 5), 1 µg (dot 6), 2 µg (dot 7). **B** Exponential regression of the corresponding dot blot signals allows determination of endogeneous VE-cadherin content to correspond to 0.076 µg VE-cadherin-Fc monomer (intersection with *x*-axis)

monomers or 5×10^3 cadherin *cis* dimers per μm^2 plasma membrane (N_A = Avogadro's number).

To determine the fraction of cadherins exposed on the cell surface (plasmalemmal fraction), we took advantage of the property of cadherins to resist trypsin proteolysis in the presence of >1 mM Ca^{2+} (Hyafil et al. 1981; Pokutta et al. 1994). Figure 3 shows an example of a series of Western blots of monolayers exposed to low and high [Ca^{2+}] in the presence and absence of trypsin. Only treatment of monolayers with trypsin in the absence of Ca^{2+} resulted in complete loss of the cadherin signal, whereas under all other conditions (low [Ca^{2+}], trypsin in the presence of high [Ca^{2+}]) immunoblotting signals remained at control levels. These experiments show that virtually all VE-cadherin molecules are inserted in the plasma membrane of cultured MyEnd cells.

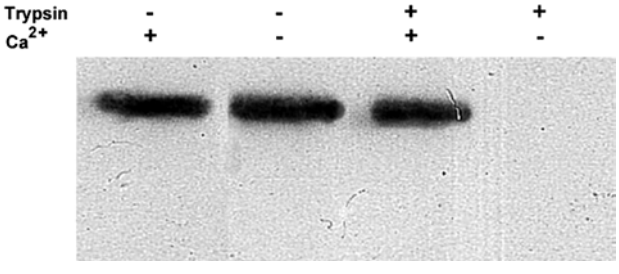

Fig. 3 Western blotting for VE-cadherin of MyEnd cell monolayers exposed to low and high [Ca^{2+}] in the absence and presence of trypsin. Complete loss of VE-cadherin signal in monolayers treated with trypsin in the absence of Ca^{2+} indicates that virtually all VE-cadherin molecules were exposed on the cell surface

Affinity (K_D) for *trans* interaction of VE-cadherin was determined by mobility shift of VE-cadherin-Fc on an affinity column loaded with covalently coupled dimeric VE-cadherin-Fc. Figure 4 shows a typical dot blot series for VE-cadherin in 120 µL fractions collected from a column loaded with soluble VE-cadherin-Fc in the presence of 2 mM Ca^{2+} and in the absence of Ca^{2+}. A typical mobility shift of VE-cadherin-Fc in the soluble phase is seen under these conditions, with a delay of mobility in the presence of Ca^{2+}. The experiments were repeated several fold on the same column (presence and absence of Ca^{2+}). During all these repetitions, VE-cadherin-Fc displayed delayed mobility only in the presence of Ca^{2+}. Additional control experiments with an affinity column covalently coupled with BSA showed the absence of any mobility shifts in the presence and absence of Ca^{2+}, further supporting our conclusion that delayed mobility seen on the VE-cadherin-Fc affinity column is caused by specific *trans* interaction with sepharose-bound VE-cadherin dimers. To confirm that binding occurs under conditions of saturation, the column was loaded with half and double amounts of soluble VE-cadherin-Fc. No significant changes in the mobility profiles were observed. Thus, the assumption used for derivation of Eq. (1) is valid, i.e. an excess of cadherins covalently linked to the solid phase that prevents saturation of *trans* interaction with cadherins in

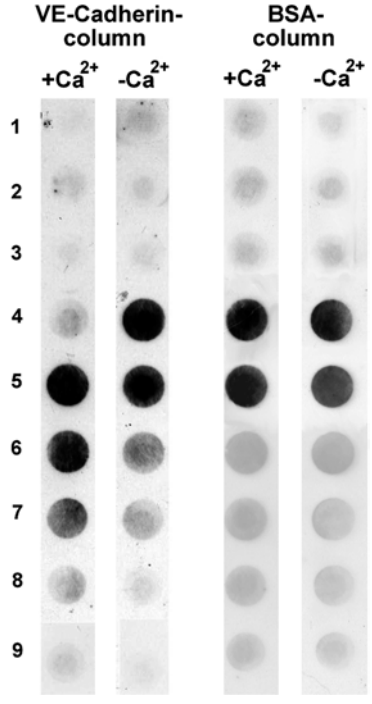

Fig. 4 Representative series of dot blots of 120 µL fractions of soluble VE-cadherin-Fc run through a VE-cadherin affinity column or BSA-coated control column, respectively, in the absence and presence of Ca^{2+}. Note comparable mobility of BSA column and Ca^{2+}-free VE-cadherin column and delayed mobility on the VE-cadherin column in the presence of 2 mM Ca^{2+}. Fraction numbers indicated on the *left side*

the soluble phase. Oligomerization or aggregation of VE-cadherin-Fc in the soluble phase did not occur. This was demonstrated by sephacryl S300 gel filtration chromatography, in which VE-cadherin-Fc migrated as a single peak and no mobility shifts were observed in the presence or absence of Ca^{2+}.

The fraction of solid phase-bound VE-cadherin-Fc accessible for *trans* interaction with VE-cadherin-Fc of the soluble phase was determined by monoclonal antibody 11D4.1, which binds to the extracellular domain of VE-cadherin and inhibits *trans* interaction. The column was loaded with hybridoma supernatant until saturation of binding (30 min at flow rate of 0.1 mL/min). Bound antibody was eluted with 10 mM citric acid (pH 2.4). No detectable amounts of VE-cadherin-Fc were eluted under these conditions. The total protein amount of eluted antibody was 0.57 mg. Assuming 1:1 stoichiometry of binding between dimeric VE-cadherin-Fc (molecular weight ~180,000) and IgG (molecular weight ~160,000), the amount of sepharose-bound VE-cadherin-Fc accessible for binding of antibody 11D4.1 was calculated to be 0.64 mg. This value is close to the total amount of VE-cadherin-Fc determined to be coupled to the column (~0.7 mg), indicating that virtually all CNBr-coupled VE-cadherin-Fc molecules are accessible for *trans* interaction with VE-cadherin-Fc in the soluble phase.

Knowledge of these data allows determination of K_D for *trans* interaction between soluble VE-cadherin-Fc and VE-cadherin-Fc bound to the solid phase. Cumulative protein content of 60 µL fractions of VE-cadherin-Fc run through the VE-cadherin affinity column in the absence of Ca^{2+} (no cadherin *trans* interaction, $K_D = \infty$) was fitted according to Eq. (2) (Fig. 5). This allowed determination of the exclusion volume and diffusion coefficient D, and thus of the velocity v and concentration c_i. For determination of K_D, these parameters were kept constant and only K_D was varied to fit the mobility curve in the presence of Ca^{2+}, yielding a K_D of 0.7×10^{-4} M for this particular experiment. Eleven experiments from three different VE-cadherin coated columns yielded an average K_D of 0.78×10^{-4} M and a 90% confidence interval ranging from 0.53×10^{-4} to 1.19×10^{-4} M, assuming logarithmic normal distribution (Papoulis 1991).

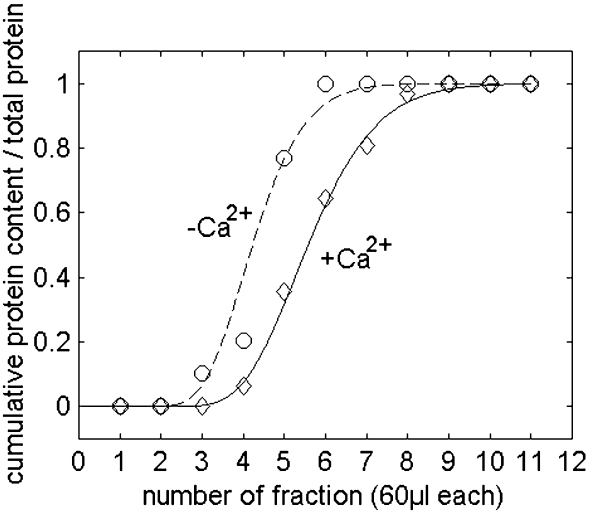

Fig. 5 Mobility shift of VE-cadherin-Fc run through a VE-cadherin affinity column in the absence and presence of Ca^{2+} (2 mM)

Discussion

In the present study, we determined both the density of VE-cadherin exposed on the cell surface of an immortalized microvascular endothelial cell line (MyEnd) and the apparent dissociation constant (K_D) for *trans* interaction of recombinant mouse VE-cadherin dimers.

Determination of the number of copies of VE-cadherin per area of the cell surface was performed by quantitative dot blot assay, in which recombinant VE-cadherin-Fc was used for calibration. Quantification of VE-cadherin in cell lysates by linear interpolation, as well as by determination of VE-cadherin content by regression analysis of dot blot signals of cell lysates to which increasing amounts of VE-cadherin-Fc monomers were added, resulted in similar values for the average content of VE-cadherin monomers per µg total endothelial protein of 12.8 and 15.2 ng, respectively. The difference of ~15% between the amount determined by both methods is statistically not significant. By trypsin cleavage of surface-exposed cadherins in the absence of Ca^{2+} we showed that virtually all VE-cadherin molecules are exposed on the cell surface of cultured MyEnd cells. This allowed us to determine the surface concentration of VE-cadherin to be about 5×10^3 dimers (10^4 monomers) per µm^2 cell surface. This value lies between the plasmalemmal concentration of the band 3 anion exchanger (AE1) in erythrocytes (10^4/µm^2) and the concentration of fibrinogen binding integrin adhesion molecules in platelets (4×10^4 copies; Eigenthaler and Shattil 1996), which can be calculated to be close to 10^3 integrins/µm^2 platelet surface (surface of 30–50 µm^2; Holmsen 1990). With respect to the average number of VE-cadherin copies per cell, MyEnd cells are supplied with about 10^7 dimers. The average circumference of individual MyEnd cells is 200 µm (10^6 cells per 25 µm^2), which corresponds to a total surface of the lateral (intercellular) cell membrane of about 600 µm^2, assuming an average depth of the lateral cell border of 3 µm. If we consider all cadherins concentrated at the interacting lateral cell surface, the local concentration could reach values of up to 5×10^4 cadherin dimers/µm^2. Considering further that each *trans* interacting cadherin dimer can transmit a unit force of 40 pN (at a separation velocity of 1 µm/s; Baumgartner et al. 2000a), this density would allow withstanding a distracting force of 2 µN/µm^2 at a separation velocity of 1 µm/s. A similar level of force-velocity relation occurs in heart muscle (20–40 mN/mm^2, contraction velocity of up to 1 µm/s; LeWinter

and Osol 2001), in which individual cardiomyocytes are mechanically connected to each other by cadherin-type adherens junctions within the intercalated discs. The distracting force acting on these junctions can be calculated to be in the range of 20–40 $nN/\mu m^2$ at 1 μm/s, and hence about two orders of magnitude below the value estimated above for the maximal capacity of force transmission of endothelial junctions. Vascular endothelial cells can also be considered to be challenged by relatively high distracting forces. These are created by blood flow (shear stress of up to 10 Pa) and by pulsatile distension of blood vessels. The plasmalemmal concentration of VE-cadherin determined in this study for MyEnd cells can be considered sufficient to withstand these considerable mechanical forces permanently acting on the endothelial layer.

These calculations on the maximally possible force transmission of junctions are based on the assumption that all cadherins of the interacting lateral cell surface undergo simultaneous adhesion (*trans* interaction) with cadherins of the apposing (contacting) cell surface. However, the surprisingly low K_D of $\sim 10^{-4}$ M determined in this study would allow only a fraction of cadherins to interact at the same time, assuming that cadherins are tethered to the cytoskeleton and are prevented from lateral diffusion. If cadherins are not immobilized by linkage to the cytoskeleton but, rather, are freely mobile in the plane of the plasma membrane, this proportion of *trans* interacting cadherins will further drop significantly (Baumgartner and Drenckhahn 2002).

For the determination of K_D, we have developed a straightforward chromatography method based on the mobility shift of a ligand passing through an affinity column. Calculation of K_D requires knowledge of the concentration of the ligands in the soluble phase and of the effective concentration of the ligands (receptors) coupled to the solid phase, i.e. the concentration of molecules that are accessible for interaction with ligands in the soluble phase. We have used a monoclonal antibody that inhibits *trans* interaction between VE-cadherins to determine the effective (accessible) concentration of sepharose-coupled cadherins. The possibility of mobility shift by self-association (dimerization, oligomerization) of cadherins in the absence or presence of Ca^{2+} was excluded by sephacryl S300 gel filtration chromatography, which did not reveal any changes in mobility under the various conditions used. The value of K_D for VE-cadherin *trans* interaction determined in the present study (assuming a single binding site per dimer) is in line with the level of K_D determined previously by us by single-molecule AFM, which was found to be in the range of 10^{-3}–10^{-5} M. Based on the present and our previous AFM data, we are now quite convinced that *trans* interaction between VE-cadherin ectodomains is an extremely low affinity reaction that would be particularly ineffective if cadherins were not immobilized by linkage to the actin filament cytoskeleton. Both the surface concentration and the K_D determined in this study are in the range that we have predicted in our theoretical approach to be effective for regulation of adhesion by the degree of cytoskeletal tethering of cadherins (Baumgartner and Drenckhahn 2002).

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