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Desmoglein 2-mediated adhesion is required for intestinal epithelial barrier integrity

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Schlegel N, Meir M, Heupel W, Holthöfer B, Leube RE, Waschke J. Desmoglein 2-mediated adhesion is required for intestinal epithelial barrier integrity. *Am J Physiol Gastrointest Liver Physiol* 298: G774–G783, 2010. First published March 11, 2010; doi:10.1152/ajpgi.00239.2009.—The integrity of intercellular junctions that form the "terminal bar" in intestinal epithelium is crucial for sealing the intestinal barrier. Whereas specific roles of tight and adherens junctions are well known, the contribution of desmosomal adhesion for maintaining the intestinal epithelial barrier has not been specifically addressed. For the present study, we generated a desmoglein 2 antibody directed against the extracellular domain (Dsg2 ED) to test whether impaired Dsg2-mediated adhesion affects intestinal epithelial barrier functions in vitro. This antibody was able to specifically block Dsg2 interaction in cell-free atomic-force microscopy experiments. For in vitro studies of the intestinal barrier we used Caco2 cells following differentiation into tight enterocyte-like epithelial monolayers. Application of Dsg2 ED to Caco2 monolayers resulted in increased cell dissociation compared with controls in a dispase-based enterocyte dissociation assay. Under similar conditions, Dsg2 antibody significantly decreased transepithelial electrical resistance and increased FITC-dextran flux, indicating that Dsg2 interaction is critically involved in the maintenance of epithelial intestinal barrier functions. As revealed by immunostaining, this was due to Dsg2 ED antibody-induced rupture of tight junctions because tight junction proteins claudins 1, 4, and 5, occludin, and tight junction-associated protein zonula occludens-1 were partially removed from cell borders by Dsg2 ED treatment. Similar results were obtained by application of a commercial monoclonal antibody directed against the ED of Dsg2. Antibody-induced effects were blocked by absorption experiments using Dsg2-Fc-coated beads. Our data indicate that Dsg2-mediated adhesion affects tight junction integrity and is required to maintain intestinal epithelial barrier properties.

tight junction

THE INTESTINAL EPITHELIAL BARRIER consists of a monolayer of columnar epithelial cells named enterocytes that are held together and sealed by circumferential intercellular junctions. The intercellular junctions form the "terminal bar," which is composed of tight junctions, adherens junctions, and desmosomes (6). Maintenance of this barrier plays a pivotal role in the protection of the organism against luminal pathogens, and thus increased paracellular permeability results in systemic contamination, potentially leading to a state of severe systemic inflammation (11, 13, 17, 24).

It is well established that tight junctions formed by claudins and occludin seal the intercellular cleft, whereas adherens junctions consisting of E-cadherin provide the mechanical strength between epithelial cells. A comparable role has been attributed to desmosomes in the epithelial intestinal barrier, where they are composed of the cadherin family adhesion proteins desmoglein 2 (Dsg2) and desmocollin 2 (Dsc2) (16, 19). However, at present there is no study that has specifically tested whether adhesion of Dsg2 is in fact required for the maintenance of the intestinal barrier. Moreover, because targeted gene deletion of Dsg2 resulted in early embryonic lethality in mice (5), a suitable knockout model to test this is not available.

Interestingly, in the skin-blistering autoimmune disease pemphigus, specific autoantibodies directed against the extracellular domain of Dsg3 block Dsg3 interaction in desmosomes of neighboring keratinocytes and thereby induce skin blistering (8, 9, 27). According to this model, we hypothesized that application of an antibody directed against the extracellular domain of Dsg2 would have similar effects on desmosomes in enterocytes, which could provide new insights into the role of Dsg2 in the maintenance of intestinal epithelial properties. Therefore, for the present study we generated an antibody directed against the extracellular domain of Dsg2 and tested whether this antibody was effective to block Dsg2 interaction in a cell-free system by using atomic-force microscopy (AFM). We established an in vitro model of the intestinal epithelial barrier to investigate whether inhibition of Dsg2 interaction would affect intestinal epithelial barrier integrity. Therefore, we used the human colon carcinoma cell line (Caco2) because these cells are known to differentiate into tight enterocyte-like monolayers dependent on cell culture conditions (18) and have therefore been extensively used as a model for the intestinal mucosa. Here, we demonstrate that application of two different antibodies directed against the extracellular domain of Dsg2 in Caco2 monolayers led to cell dissociation and decreased intestinal epithelial barrier properties. Moreover, under these conditions tight junction proteins at the cell borders were reduced, which suggested that desmosomal adhesion is required for epithelial tight junction integrity.

MATERIALS AND METHODS

Cell culture. Caco2 cells were acquired from the Deutsche Sammlung für Mikroorganismen und Zellkulturen (Braunschweig, Germany) and were cultured in DMEM (Life Technologies, Karlsruhe, Germany) supplemented with 50 U/ml penicillin-G, 50 μg streptomycin, and 10% fetal calf serum (FCS) (Biochrom, Berlin, Germany) in a humidified atmosphere (95% air-5% CO_2) at 37°C. Cultures were used for experiments when grown to confluent monolayers as indicated below. Stably transfected Chinese hamster ovary cells secreting Dsg2-Fc (see *Recombinant Dsg2-Fc*) were grown in DMEM supplemented with 10% FCS and antibiotics at 37°C in humidified atmosphere (5% CO_2).

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Generation and characterization of Dsg2 antibodies. With the use of a murine Dsg2 cDNA template in plasmid pCAGGmDsg2 (kindly provided by Dr. David Garrod, University of Manchester, UK.), two fragments were amplified by PCR with primer pairs dsg2-AK5-f 5′-GGC AGC CTC GAG GAA GAC GTC AAT GAC AAT TG-3′/dsg2-AK5-r 5′-GGC AGC CTC GAG CAA CCC GAC GTA GTT GTC-3′ and dsg2-AK3-f 5′-GGC AGC CTC GAG CGA AAG ATC GAT CTG GAT G-3′/dsg2-AK3-r 5′-GGC AGC CCT CGA GGG AGT AAG AAT GTT GCA T-3′. The amplified fragments encode the extracellular anchor domain and fourth extracellular domain (EC4) or the cytoplasmic carboxyterminus starting at the intracellular linker domain, respectively. Sequence comparisons suggested that these regions are suitable for isoform-specific antibody generation against the extracellular anchor domain and EC4 of Dsg2 (Dsg2 ED) or against the intracellular domain of Dsg2 (Dsg2 IC), respectively. The XhoI-cleaved PCR fragments were therefore inserted into the bacterial expression vector pET15b (Novagen, Madison, WI). With the use of standard protocols, the His-tagged recombinant polypeptides were enriched from bacterial lysates by nickel agarose chromatography and subsequently buffered in PBS. Antibodies were generated in adult New Zealand rabbits by subcutaneous injection of 100 μg of purified Dsg2 polypeptide mixed with complete Freund's adjuvant followed by two boosting injections of 100 μg of antigen in incomplete Freund adjuvant after 4 wk. Serum was collected 10 days after the last immunization. Serum and antibodies were stored at −20°C. Thereafter, Western blot assays, immunostaining, and AFM were used to characterize the Dsg2 antibodies. For specificity studies we additionally used a commercially available antibody directed against the second and third extracellular repeat domain of Dsg2 (clone 10G11; Acris, Herford, Germany). The antibody was reconstituted in distilled H_2O as recommended by the manufacturer. For all experiments on intestinal epithelial barrier regulation the Dsg2 ED antibodies were diluted 1:20 in HBSS or PBS, respectively.

Cytochemistry. Immunostaining has been described in detail previously (21). In brief, epithelial cells were grown to confluence on cover slips as indicated below. After incubation with or without different mediators as indicated below, cells were fixated with 2% formaldehyde for 10 min at room temperature. After treatment with 0.1% Triton X-100 for 15 min, monolayers were incubated at 4°C overnight using the following primary antibodies (1:100 each in PBS): mouse monoclonal E-cadherin antibody (clone 36; BD Biosciences, Heidelberg, Germany), mouse monoclonal Dsg1 antibody (clone Dsg1-P124; Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal Dsg2 antibody (clone 10G11; Acris), mouse monoclonal Dsg3 antibody (clone 5G11; Zymed, San Francisco, CA), rabbit polyclonal occludin antibody (Zymed/Invitrogen, Carlsbad, CA), rabbit polyclonal antibodies directed against claudin 1, 2, 3, 4 or 5, respectively (all Zymed/Invitrogen Carlsbad), and rat polyclonal zonula occludens (ZO)-1 antibody (Zymed/Invitrogen). As secondary antibodies we used Cy3-labeled goat anti-mouse, goat anti-rabbit, or goat anti-rat, respectively (diluted 1:600 in PBS; Dianova, Hamburg, Germany). Cover slips were mounted on glass slides with 60% glycerol in PBS, containing 1.5% *N*-propyl gallate (Serva, Heidelberg, Germany) as antifading compound. Representative experiments were photographed with a confocal microscope (LSM 510; Carl Zeiss Microimaging, Göttingen, Germany).

Western blotting. SDS-PAGE and immunoblotting were carried out as described previously (22). Antibodies against E-cadherin, Dsg2, occludin, claudin 1, and ZO-1 (see above) were used at a dilution of 1:500. As secondary antibodies, the respective horse-

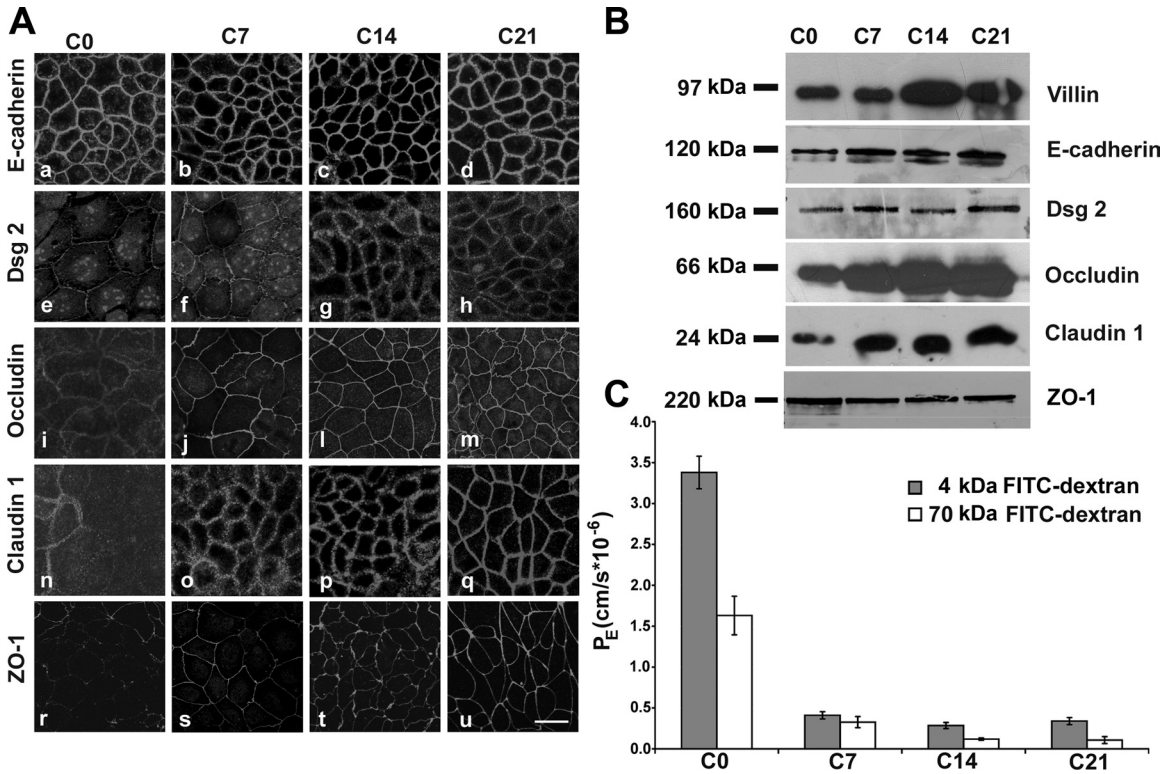

Fig. 1. Maturation of intestinal epithelial barrier properties in Caco monolayers was completed after 14 days of confluence. *A*: immunostaining was performed for adherens junctions protein E-cadherin (*a–d*), desmoglein 2 (Dsg2) (*e–h*), tight junction proteins occludin (*i–m*), claudin 1 (*n–q*), and zonula occludens (ZO)-1 (*r–u*) at 0 (C0; *a*, *e*, *i*, *n*, and *r*), 7 (C7; *b*, *f*, *j*, *o*, and *s*), 14 (C14; *c*, *g*, *l*, *p*, and *t*), and 21 (C21; *d*, *h*, *m*, *q*, and *u*) days after Caco2 monolayers had grown to confluence. Experiments shown are representatives for $n \geq 5$; scale bar is 50 μm. *B*: Western blot analyses were used to document alterations in total protein amounts of villin, E-cadherin, Dsg2, occludin, claudin 1, and ZO-1 in the time course of differentiation of Caco monolayers from C0–C21. Western blots shown are representatives for $n \geq 4$ for each condition. *C*: measurements of 4-kDa and 70-kDa FITC-dextran flux across Caco2 monolayers were measured at C0-C21 to assess functional alterations of barrier properties ($n \geq 6$ for each condition).

radish peroxidase-labeled goat anti-rabbit IgG, goat anti-mouse, or goat anti-rat IgG (Santa Cruz, Heidelberg, Germany) were used (1:3,000 in PBS). Bound immunoglobulins were visualized by the enhanced chemiluminescence technique (ECL, Amersham, Braunschweig, Germany).

Measurement of FITC-dextran flux across monolayers of cultured epithelial cells. As described previously (21), epithelial cells were seeded on top of Transwell chambers for six-well plates (0.4 μm pore size) (Falcon, Heidelberg, Germany) and grown to confluence. After being rinsed with PBS, cells were incubated with fresh DMEM without phenol red (Sigma, St. Louis, MO) containing 10 mg/ml FITC-dextran (4 kDa and 70 kDa, Sigma) in the presence or absence of different mediators as indicated below. Paracellular flux was assessed by taking 100-μl aliquots from the outer chamber over 2 h of incubation. Fluorescence was measured using a Wallac Victor 2 fluorescence spectrophotometer (Perkin-Elmer, überlingen, Germany) with excitation and emission at 485 and 535 nm, respectively. For all experimental conditions permeability coefficients (P_E) were calculated by the following formula (21): $P_E = [(\Delta C_A/\Delta t) \times V_A]/(S \times \Delta C_L)$, where P_E = diffusive permeability (cm/s), ΔC_A = change of FITC-dextran concentration, Δt = change of time, V_A = volume of the abluminal medium, S = surface area, ΔC_L = constant luminal concentration.

Measurement of transepithelial resistance. ECIS 1600R (Applied BioPhysics, Troy, NY) was used to measure transepithelial resistance (TER) of epithelial monolayers as described previously for endothelial cells (20, 22). Epithelial cells were seeded in the wells of the electrode array and grown to confluence as indicated below. Then medium was exchanged (200 μl), and baseline TER was measured for 10 min to equilibrate monolayers. Afterward, 200 μl of medium containing different mediators or medium without mediators was applied to the wells.

Recombinant Dsg2-Fc. The complete extracellular Dsg2 domain including the endoplasmic reticulum signal sequence was amplified using 5′-ggatcagctagccaccatggcgcggagcccgggacgcgcgt-3′ and 5′-ccgctcgaggcccacataggagtcatgctgtgct-3′ (introduced *NheI* and *XhoI* restriction sites are underlined) primers and cloned in frame with the cDNA encoding the Fc-fragment of human IgG 1 (including the hinge region and Ig domains CH2 and CH3) using *NheI/XhoI*-digested plasmid pEGFP-Cad11-Fc (10). Chinese hamster ovarian cells were transfected with pEGFP-Dsg2-Fc using Effectene transfection reagent (Qiagen, Hilden, Germany) following the manufacturer's protocol. After selection of cells by application of 1.4 mg/ml geneticin and several subcloning steps, stable transfected cell clones secreting Dsg2-Fc in the cell culture supernatant were isolated. Individual clones were characterized for production of the fusion protein by immunostaining with Cy3-labeled goat-anti-human IgG. For purification of Dsg2-Fc, cells were seeded on Petri dishes (140 cm^2) and cultured with DMEM only. One liter of cell culture supernatant was collected, and after addition of protease inhibitors (aprotinin, pepstatin, leustatin; 1 mg/ml each) the supernatant was centrifuged for 25 min at 10,000 *g* and 4°C. Purification of Dsg2-Fc was done by affinity chromatography using protein A agarose (Oncogene, Cambridge, MA). The protein was eluted by citrate buffer (25 mM, pH 2.4), immediately dialyzed against HBSS (Applichem, Darmstadt, Germany) for 16 h at 4°C and stored in aliquots at −80°C. Protein content was determined using Bradford method, and purity was checked by Coomassie Blue staining of 7.5% SDS-PAGE.

Antibody absorption experiments. For antibody absorption experiments, magnetic nickel beads (Invitrogen, Karlsruhe, Germany) were coated with Dsg2-Fc-protein according to the manufacturer's instructions. In brief, 50 μl of the beads were washed twice in HBSS. Thereafter, beads were incubated with 10 μg of Dsg2-Fc protein in 700 μl HBSS for 10 min at room temperature on a rotator. Finally beads were washed four times in HBSS. Absorption experiments were carried out as follows: Dsg2 antibodies directed against the ED (polyclonal or monoclonal, respectively) were diluted 1:20 in HBSS. Thereafter, Dsg2-Fc-coated beads were added and left on a rotator at for 30 min at room temperature. Beads were removed using a magnet, and successful absorption of the Dsg2 antibodies was verified by Western blot analyses.

AFM. Dsg2 transinteraction was characterized by force-distance measurements using a Bioscope AFM driven by a Nanoscope III controller (Digital Instruments, Santa Barbara, CA) as described in detail recently (9). Recombinant Dsg2-Fc containing the complete ED of Dsg2 was linked covalently to the Si_3N_4 tips of the cantilever (Veeco instruments, Mannheim, Germany) and freshly cleaved mica plates (Spi supplies, West Chester, PA) using polyethylene glycol (PEG) spacers containing an aminoreactive cross-linker group (N-hydroxy-succinimide ester) at one end and an aldehyde group at the other end. Force measurements were performed in buffer A containing 10 mM HEPES, 150 mM NaCl, pH 7.4 and 2 mM Ca^{2+}. Unbinding forces between molecules coupled to the tip and plate of the AFM

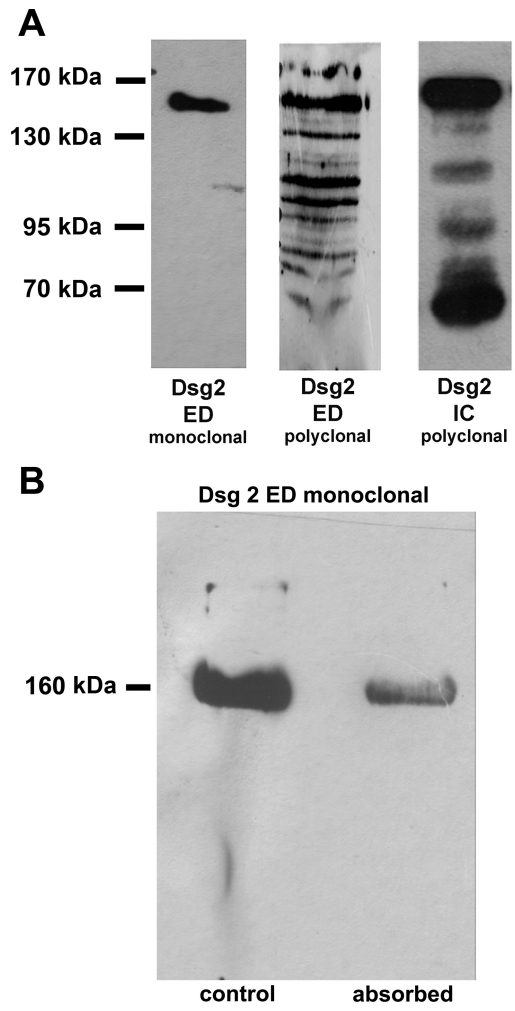

Fig. 2. Dsg2 antibodies were tested by Western blotting. *A*: commercially available antibody directed against the extracellular domain of Dsg2 (Dsg2 ED) showed a single Dsg2-specific band at 160 kDa (*lane 1*). The self-generated antibodies directed against Dsg2 ED (*lane 2*) and against the intracellular domain of Dsg2 (Dsg2 IC) (*lane 3*) also showed a strong band at 160 kDa. However, both of these antibodies also showed a number of unspecific bands at lower molecular weights (Western blots shown are representatives for $n = 3$ experiments). *B*: postabsorbed antibody dilutions were incubated with Western blot membranes that were loaded with 15 μg of Dsg2-purified protein. Under these conditions the specific band for Dsg2 was significantly weaker than in controls that were incubated with Dsg2 ED monoclonal without absorption (Western blot is representative for $n = 3$ experiments).

were monitored by force-distance cycles at amplitudes of 500 nm and 1-Hz frequency. For each condition 500 approach-retrace cycles were recorded using an individual AFM tip. Experiments were performed in triplicate using different AFM tips. Binding frequency of homophilic transinteraction was evaluated by analyzing and counting specific unbinding events with a Dsg2-coated tip in force-distance cycles under different conditions, thereby reflecting both effective concentrations of Dsg2 molecules on the tip and binding probabilities. For Ca^{2+} depletion, buffer A supplemented with 4 mM EGTA was used. Antibodies were applied at 1:10 dilution.

Dispase-based enterocyte dissociation assay. The assay was performed as described previously for keratinocytes with some modifications (3, 8). In brief, Caco2 cells were seeded on 12-well plates and grown to confluence. After 14 days of confluence, cells were incubated for 24 h under various conditions as indicated below. Thereafter, cells were washed with HBSS and treated for 30 min with 0.3 ml dispase II (2.4 U/ml, Sigma) at 37°C. Afterward, dispase solution was carefully removed and cells dissolved in 0.5 ml HBSS. Mechanical stress was then applied by pipetting 10 times with a 1-ml pipette. Finally, dissociation was quantified by counting and averaging cell fragments in three defined areas of each condition under a binocular microscope. Every condition was performed in quadruplicate and repeated five times.

Statistics. Values throughout are expressed as means ± SE. Possible differences of were assessed using unpaired Student's *t*-test and nonparametric Mann-Whitney statistic. Statistical significance is assumed for $P < 0.05$.

RESULTS

Maturation of barrier properties in Caco2 cell cultures was completed after 14 days. We first characterized maturation of barrier properties in Caco2 epithelial monolayers to assess the most reliable conditions for in vitro analyses of intestinal barrier functions. As described in MATERIALS AND METHODS, Caco2 cells were seeded and barrier properties were compared after 0 days (C0), 7 days (C7), 14 days (C14), and 21 days (C21) of confluence.

Immunostaining demonstrated that heterogenous Caco2 monolayers at C0 had changed to homogenous monolayers within 14 days (C14) of confluence. Adherens junction protein E-cadherin (Fig. 1*A*, *a–d*) and Dsg2 (Fig. 1*A*, *e–h*) were regularly distributed along cell borders, whereas positive immunostaining for Dsg1 and 3 was never observed (not shown). Tight junction proteins occludin (Fig. 1*A*, *i–m*), claudin 1 (Fig. 1*A*, *n–p*), and ZO-1 (Fig. 1*A*, *r–u*) were hardly visible in most cells at C0, were more obvious at C7, and were finally regularly distributed at cell borders at 14 and 21 days after

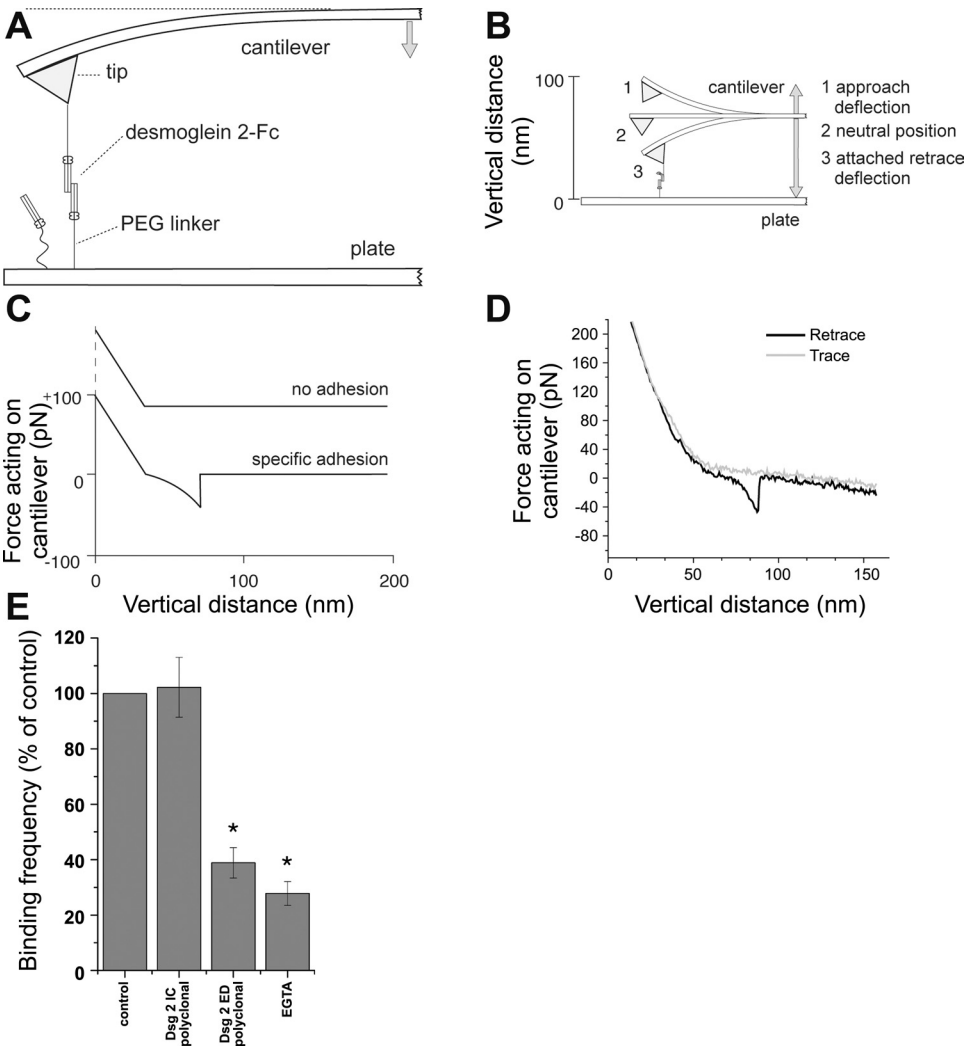

Fig. 3. Determination of Dsg2 single molecule binding frequencies by atomic-force microscopy (AFM) studies. *A*: binding frequencies of Dsg2 fusion proteins covalently coupled to the tip and plate of the AFM via flexible polyethylene glycol (PEG) linker were monitored by force-distance cycles. *B*: molecules were brought into contact by repeated downward (trace) and upward movement (retrace) of the AFM tip. *C*: during upward movement, a downward deflection of the cantilever occurred if plate- and tip-bound Dsg molecules underwent specific transinteractions. After reaching a critical force, the bond broke and the cantilever jumped back to the neutral position. *D*: in a sample force-distance plot a characteristic unbinding event of Dsg2-Fc molecules is shown. *E*: bar graph shows that Dsg2 ED significantly reduced binding activity to 39 ± 5%. Ca^{2+} depletion using EGTA reduced binding activity to 28 ± 4%. Dsg2 IC antibody did not alter binding activity (every experiment was performed in triplicate and repeated for 5 times; *significant difference $P < 0.05$ compared with controls).

confluence, suggesting the presence of mature tight junctions at these time points.

In Western blot analyses (Fig. 1*B*) total protein amount of enterocyte marker protein villin increased, especially in the time between C7 and C14. E-cadherin and Dsg2 augmented only slightly from C0 to C21. In contrast, Dsg1 and Dsg3 were not detected in Caco2 cell lysates (not shown). According to the immunostaining studies for tight junction proteins, a strong increase of total protein amounts of occludin and claudin 1 was detected from C0 to C21, whereas ZO-1 remained unchanged.

Calculation of the permeability coefficients (P_E) of 70-kDa and 4-kDa FITC-dextran fluxes across epithelial monolayers was used for functional analyses of barrier properties of Caco2 monolayers (Fig. 1*C*). At C0 P_E was 1.6 ± 0.23 cm/s $\times 10^{-6}$ for 70-kDa FITC dextran, whereas P_E was significantly higher for 4-kDa FITC-dextran (3.3 ± 0.19 cm/s $\times 10^{-6}$). After 1 wk (C7) P_E was significantly reduced for both 4-kDa and 70-kDa FITC-dextran. After 14 days of confluence (C14) P_E was significantly reduced to 0.11 ± 0.01 cm/s $\times 10^{-6}$ for 70-kDa FITC-dextran and to 0.28 ± 0.04 cm/s $\times 10^{-6}$ for 4 kDa. This was not significantly altered at C21. At C14 TER was 92 ± 0.2 $\Omega \cdot cm^2$, which was comparable to colon mucosal resistance measured in adult rats ex vivo (1). In summary, these data demonstrated that barrier properties of Caco monolayers were fully differentiated after 14 days of confluence. Therefore, these conditions were chosen for all following experiments.

Dsg2 antibodies directed against the ED specifically blocked Dsg2 interaction in a cell-free system. In Western blot analyses, we compared the self-generated antibodies with a commercially available Dsg2 antibody that is directed against the Dsg2 ED monoclonal (Fig. 2*A*). The commercial Dsg2 ED monoclonal antibody constantly detected the Dsg2-specific band 160 kDa in Caco2 lysates. Similarly, Western blots after incubation with the self-generated antibodies directed against the Dsg2 ED polyclonal and against the Dsg2 IC polyclonal showed a strong band at 160 kDa. However, the latter polyclonal antibodies also showed a number of bands at lower molecular weights. This may be explained by cross reactivity of the antibodies with other antigens or alternatively by recognition of the differentially glycosylated Dsg2 proteins.

To confirm that the observed effects in this study were due to antibody-induced disruption of Dsg2 interaction, we performed antibody absorption experiments. For these experiments, Dsg2 ED polyclonal and Dsg2 ED monoclonal antibody dilutions (1:20) were incubated with Dsg2-Fc-coated nickel beads to allow binding of the antibodies to the beads. Thereafter, Dsg2-Fc-coated nickel beads were removed using a magnet, which resulted in "postabsorbed" antibody dilutions that were used for the following experiments. To verify successful absorption of the antibodies, the postabsorbed dilutions were used for Western blot analyses (shown for the monoclonal antibody in Fig. 2*B*). Controls were incubated with the respective antibody dilutions without absorption. After absorption of the antibodies by Dsg2-Fc-coated nickel beads, the Western blot band was considerably weaker than under control conditions, indicating that this approach was effective to significantly remove the Dsg2 ED antibodies from the respective dilutions.

In a next step, AFM was used to address whether Dsg2 ED polyclonal antibody specifically recognizes and binds to the Dsg2 ED. As a proof of principle these experiments also served to study whether such antibody was capable to block Dsg2 interaction in a cell-free system (Fig. 3). Binding frequencies of Dsg2 fusion proteins covalently coupled to the tip and plate of the AFM via flexible PEG linker were monitored by force-distance cycles (Fig. 3*A*). The molecules were brought into contact by repeated downward (trace) and upward movement (retrace) of the AFM tip (Fig. 3*B*). During upward movement, a downward deflection of the cantilever occurred if plate- and tip-bound Dsg2 molecules underwent specific transinteractions (Fig. 3*C*). After reaching a critical force, the bond broke and the cantilever jumped back to the neutral position. Treatment with Dsg2 ED resulted in significantly reduced binding activity ($39 \pm 5\%$ of controls; Fig. 3*E*), indicating the specificity of both Dsg2 interaction and the Dsg2 ED in our AFM setup. Comparable results were obtained after Ca^{2+} depletion by using EGTA, which reduced binding activity to $28 \pm 4\%$. In contrast, the Dsg2 antibody directed against the Dsg2 IC did not alter binding activity ($102 \pm 1\%$ of controls). These results demonstrated that Dsg2 ED polyclonal antibody specifically binds to Dsg2 EDs and is capable to disrupt Dsg2 interactions in cell-free experiments.

Dsg2 ED antibodies increased number of fragments in cell-dissociation assays. To determine the role of Dsg2-mediated interaction for intercellular adhesion, we performed enterocyte-dissociation assays. Caco monolayers were incubated in the absence (control) or presence of Dsg2 ED polyclonal and Dsg2 ED monoclonal for 24 h (1:20). After incubation with dispase, cell monolayers were mechanically stressed by pipetting 10 times, and monolayer fragments were counted. In controls the mean number of fragments/area was 8 ± 1. Incubation of Caco2 monolayers with Dsg2 ED polyclonal for 24 h resulted in a significantly increased number of fragments/area (6 ± 0.5-fold of controls; Fig. 4). Similar results were obtained after incubation with Dsg2 ED monoclonal, which significantly increased the number of fragments/area to 5.5 ± 0.5-fold compared with controls. Incubation of monolayers with EGTA as positive controls led to 32 ± 7-fold increased number of fragments/area compared with controls.

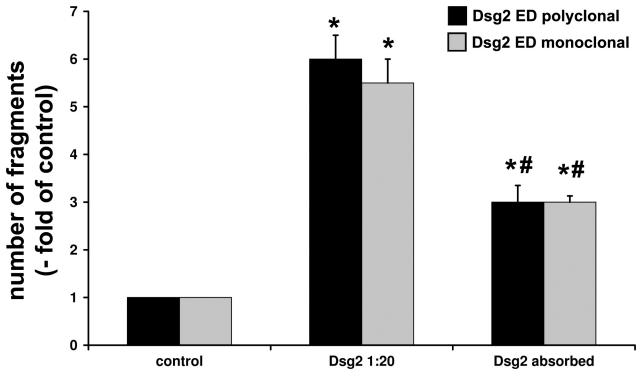

Fig. 4. Dsg2 ED antibodies significantly increased cell dissociation in Caco2 monolayers. Incubation of Caco monolayers with either polyclonal (solid column) or monoclonal (shaded column) Dsg2 ED antibodies for 24 h significantly increased cell dissociation to 6 ± 0.5-fold and to 5.5 ± 0.5-fold of controls, respectively. Treatment of the monolayers with postabsorbed antibody dilutions only increased the number of fragments to 3 ± 0.4-fold and to 3 ± 0.1-fold of controls, respectively, which was significantly reduced compared with antibody treatment alone (*$P < 0.05$ compared with controls; #$P < 0.05$ compared with antibody treatment).

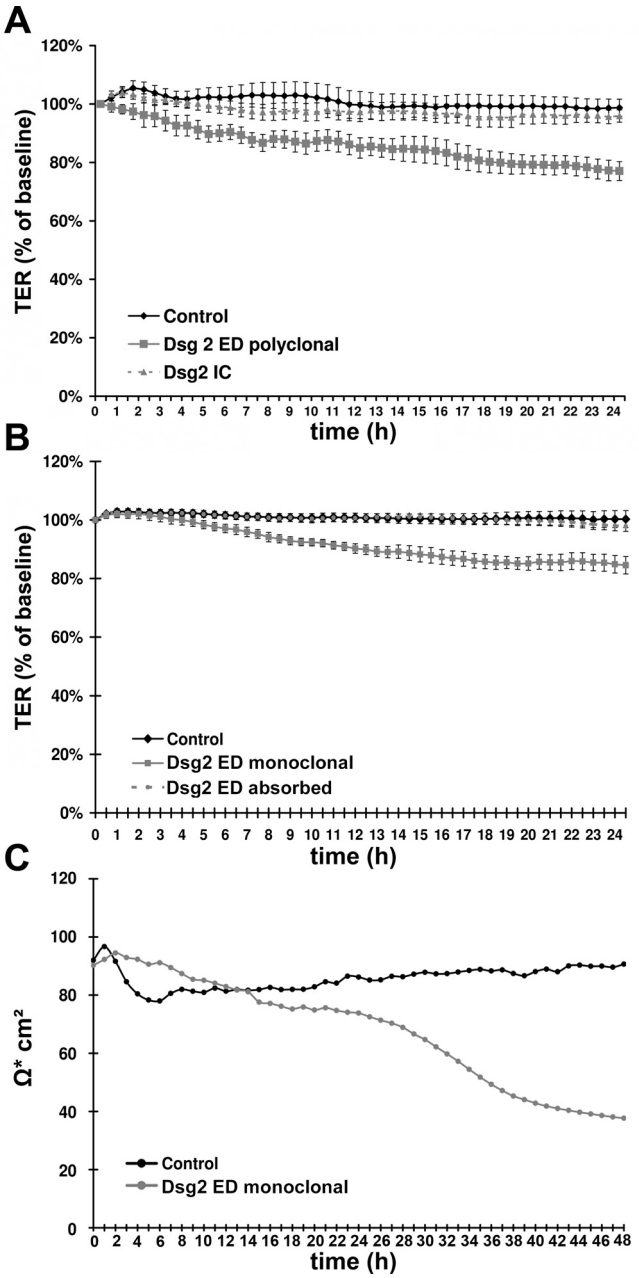

Fig. 5. Dsg2 ED antibody significantly decreased transepithelial electrical resistance (TER) across Caco2 monolayers. *A*: application of Dsg2 ED polyclonal antibody (1: 20) induced significant loss of barrier properties (77 ± 3% of baseline; $n = 7$) compared with controls (97 ± 3% of baseline; $n = 10$) in TER measurements. In contrast, incubation of monolayers with Dsg2 IC antibody did not change TER compared with controls (96 ± 2%; $n = 9$). *B*: application of Dsg2 ED monoclonal antibody (1:20) resulted in significant loss of TER within 24 h (85 ± 3% after 24 h; $n = 6$). Similar to controls ($n = 5$), treatment of monolayers with postabsorbed antibody dilution did not change TER at all (98 ± 2% after 24 h; $n = 10$). *C*: representative long-term experiment over 48 h is shown after application of Dsg2 ED monoclonal antibody. Although the control experiment largely remains at baseline levels, a dramatic loss of TER can be seen after application of Dsg2 ED antibody.

Incubation of Caco2 monolayers with postabsorbed antibody dilutions augmented the number of fragments to 3 ± 0.4-fold for Dsg2 ED polyclonal and to 3 ± 0.1-fold of controls for Dsg2 ED monoclonal in the enterocyte-dissociation assay, respectively (Fig. 4).

Dsg2 interaction is required for maintenance of epithelial barrier properties. TER was measured to test whether application of Dsg2 ED antibodies would affect epithelial barrier integrity. Because baseline resistance in different experiments ranged from 70 to 110 $\Omega \cdot cm^2$, the baseline TER of each experiment was set as 100% to enable comparison and statistical analysis of the changes during the time course of measurements. Application of Dsg ED polyclonal antibody significantly (1:20) decreased TER to 77 ± 3% of baseline within 24 h, whereas controls remained unchanged (97 ± 3% after 24 h) during the time course of measurements (Fig. 5*A*). In contrast, treatment of monolayers with Dsg2 IC antibody did not alter TER significantly compared with controls (Fig. 5*A*). Application of Dsg2 ED monoclonal antibody significantly decreased TER to 85 ± 3% after 24 h (Fig. 5*B*). Incubation of the monolayers with postabsorbed antibody dilution as described above was not sufficient to significantly change TER (98 ± 2% after 24 h, Fig. 5*B*). Representative experiments of absolute TER values are shown in Fig. 5*C* to demonstrate the alterations of TER in the time course in 48 h after application of Dsg2 ED monoclonal antibody.

Next, we performed 4-kDa FITC-dextran flux measurements after treatment of Caco2 monolayers with Dsg2 ED monoclonal antibodies for 24 h (Fig. 6). Under control conditions P_E was 0.37 ± 0.05 cm/s × 10^{-6}. After incubation with Dsg2 ED for 24 h P_E significantly increased to 1.78 ± 0.20 cm/s × 10^{-6}.

Dsg2-mediated adhesion is required for tight junction integrity. Immunostaining revealed that, in controls, all junctional proteins (Fig. 7, *a*, *e*, *i*, *m*, and *q*) were regularly distributed along the cell borders of C14 Caco2 monolayers. Application of either Dsg2 ED polyclonal and Dsg2 ED monoclonal antibodies (1:20) for 24 h led to a reduction of tight junction proteins occludin (Fig. 7, *b* and *c*), claudin 1 (Fig. 7, *f* and *g*), and ZO 1 (Fig. 7, *j* and *k*) at the cell borders in most parts of the Caco2 monolayers as exemplified in Fig. 6. Whereas claudin 2 and claudin 3 were not detected by immunostaining of Caco2 monolayers (Fig. 8, *a* and *b*), claudin 4 and claudin 5 were also removed from the cell borders by Dsg2 ED

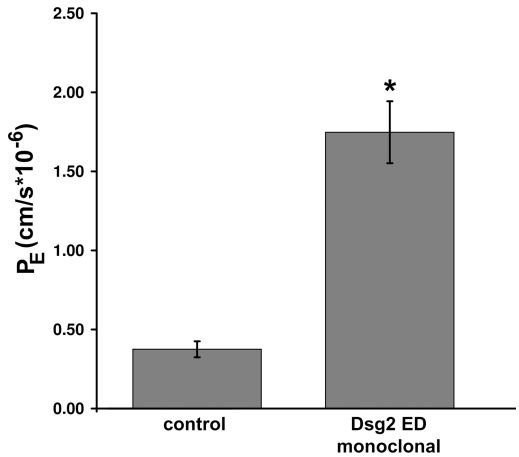

Fig. 6. Application of Dsg2 ED antibody increased FITC-dextran flux across Caco2 monolayers. Measurements of 4-kDa FITC-dextran flux across Caco2 monolayers were performed with and without application of Dsg2 ED monoclonal antibody 1:20 for 24 h. Permeability coefficient (P_E) was calculated and amounted 0.37 ± 0.05 cm/s × 10^{-6} under control conditions ($n = 17$). Incubation of monolayers with Dsg2 ED significantly augmented P_E 1.78 ± 0.20 cm/s × 10^{-6} ($n = 6$; $^*P \leq 0.05$ compared with controls).

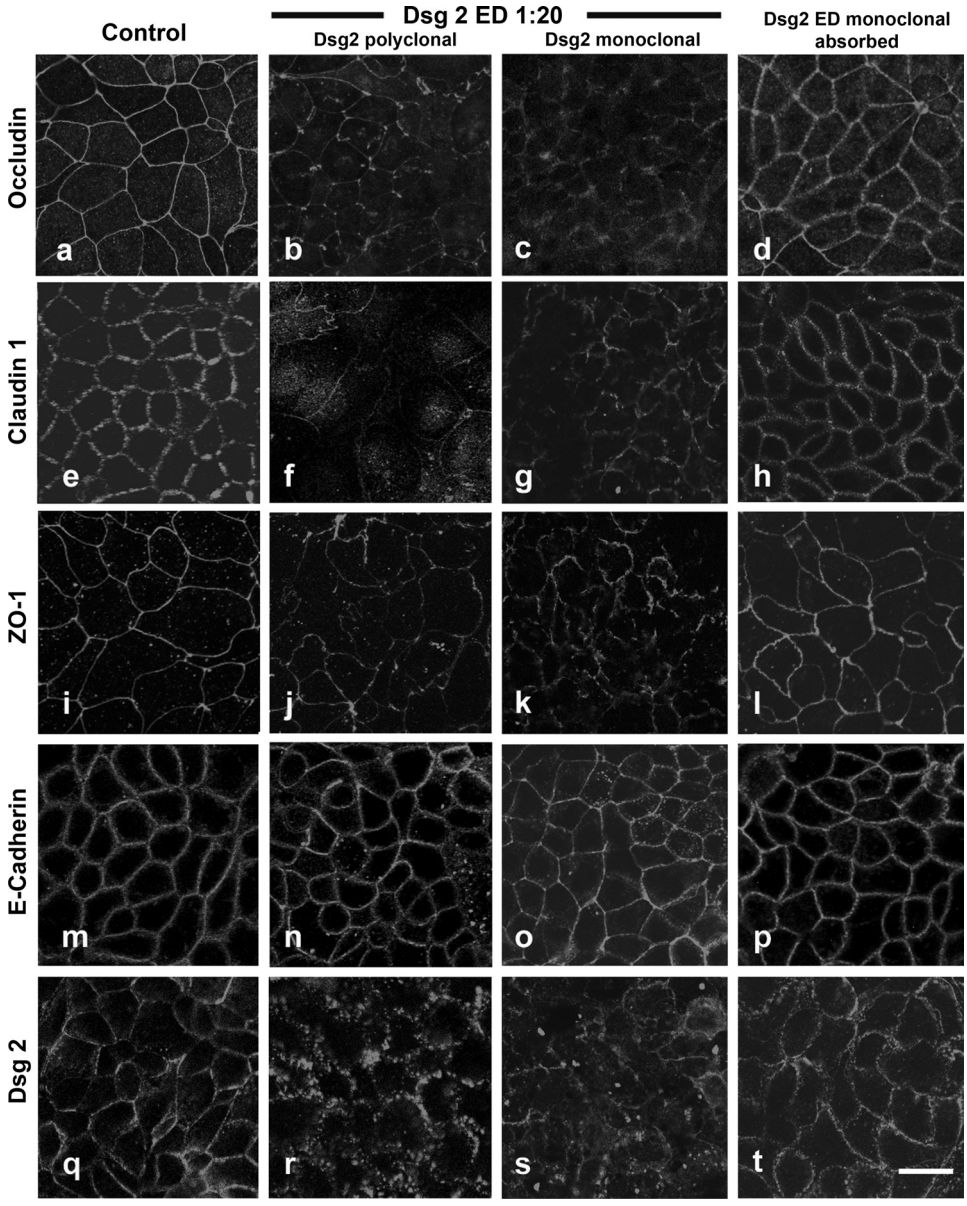

Fig. 7. Dsg2 ED antibody reduced tight junction staining at the cell borders. Immunostaining was performed to document alterations after application of Dsg2 ED (1:20) antibody for 24 h. In controls tight junction proteins occludin (*a*), claudin 1 (*e*), tight junction-associated protein ZO-1 (*i*), adherens junction protein E-cadherin (*m*), and Dsg2 (*q*) were regularly distributed at the cell borders within C14 Caco2 monolayers. Application of Dsg2 ED polyclonal or monoclonal for 24 h resulted in significantly reduced staining of occludin (*b* and *c*), claudin 1 (*f* and *g*), and ZO-1 (*j* and *k*) in most parts of the monolayers, whereas E-cadherin (*n* and *o*) staining was unaltered. Dsg2 was fragmented at the cell borders (*r* and *s*). All of these changes were not detectable when monolayers were treated with postabsorbed antibody dilutions (*d*, *h*, *l*, *p*, and *t*). Experiments shown are representatives for $n \geq 4$; scale bar is 20 μm.

polyclonal in most parts of the monolayers (Fig. 8, *g* and *h*). However, E-cadherin staining (Fig. 7, *n* and *o*) was not affected at all, indicating that the observed effects of Dsg2 antibodies were specific for tight junctions. Dsg2 staining was fragmented at the cell borders after incubation with Dsg2 ED antibodies (Fig. 7, *r* and *s*). Incubation of monolayers with postabsorbed antibody dilutions from Dsg2 ED monoclonal did not alter staining patterns of any of the proteins studied (Fig. 7 *d*, *h*, and *t*), indicating that the observed effects were specifically induced in response to altered Dsg2 interaction.

Western blot analyses were performed to test whether loss of tight junction proteins at the cell borders would lead to a reduction of total protein amounts of either claudin 1 and occludin (Fig. 9). This, however, was not the case because claudin 1 (Fig. 9*A*) and occludin-specific bands (Fig. 9*B*) in Western blot analyses were unaltered following Dsg2 ED monoclonal antibody treatment (1:20) for 24 h in all experiments.

In summary these data demonstrated that inhibition of Dsg2-mediated adhesion by using two different antibodies directed against the Dsg2 ED significantly decreased epithelial barrier properties and that Dsg2-mediated adhesion appears to be required for maintenance of tight junction integrity.

DISCUSSION

In the present study, we tested the role of Dsg2-mediated adhesion for maintenance of intestinal epithelial barrier properties in an in vitro model. We demonstrate that Caco2 monolayers developed a mature epithelial barrier within 14 days after confluence. To test the role of Dsg2-mediated adhesion for intestinal epithelial barrier integrity, we generated an antibody directed against the Dsg2 ED and additionally used a commercially available Dsg2 antibody that is also directed against the Dsg2 ED. We demonstrate that the self-generated Dsg2 antibody is able to bind to the Dsg2 ED and to specifi-

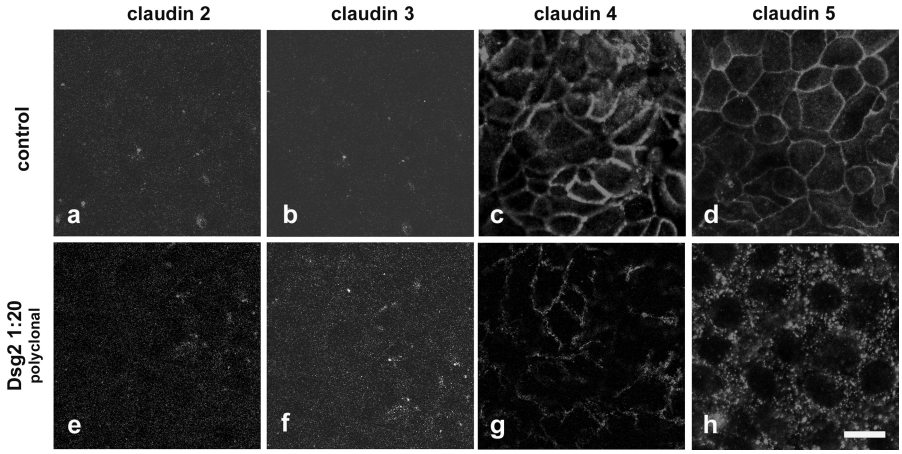

Fig. 8. Claudin 4 and claudin 5 were removed from cell borders by Dsg2 ED treatment. Immunostaining was performed for tight junction proteins claudin 2 (*a* and *e*), claudin 3 (*b* and *f*) claudin 4 (*c* and *g*), and claudin 5 (*d* and *h*) under control conditions (*a–d*) and after application of Dsg2 ED polyclonal for 24 h (*e–h*). Although no positive staining for claudin 2 (*a* and *e*) and claudin 3 (*b* and *f*) was detected, claudin 4 (*g*) and claudin 5 (*h*) were partially removed from the cell borders after Dsg2 ED treatment. Experiments shown are representatives for $n = 3$; scale bar is 20 μm.

cally block Dsg2 interaction in cell-free AFM experiments. In a dispase-based assay, both Dsg2 ED antibodies were effective in Caco2 monolayers to cause cell dissociation. TER and FITC-dextran flux measurements revealed that Dsg2 ED antibodies significantly decreased barrier properties, indicating that Dsg2-mediated adhesion was required for maintenance of intestinal epithelial barrier functions. Finally, incubation with Dsg2 ED antibodies was followed by tight junction rupture as shown by fragmented immunostaining of tight junction proteins claudin 1, occludin, and tight junction-associated protein ZO-1 along cell borders in most parts of the monolayer. Similarly, Dsg2 staining at the cell borders was fragmented, whereas E-cadherin staining was unaffected. All of these effects were not observed when Caco2 monolayers were incubated with postabsorbed dilutions where Dsg2 ED antibodies were absorbed by using Dsg2-Fc-coated beads. Similarly, no effects on intestinal epithelial barrier properties were observed after application of an antibody directed against Dsg2 IC.

Caco2 monolayers represent a suitable model for the intestinal epithelial barrier. Although Caco2 cells originate from human colon adenocarcinoma, they have extensively been used as a model for the intestinal barrier in previous studies (18). In general, it has been reported that, dependent on cell culture conditions, Caco2 cells postconfluence differentiate into polarized cells that closely resemble enterocytes in morphology and function. To assess the most reliable conditions that allow comparisons with an intact intestinal epithelial barrier, we carefully characterized the differentiation of barrier properties of the Caco2 monolayers in the present study. We demonstrate that epithelial barrier properties of Caco monolayers were fully differentiated after 14 days of confluence as revealed by immunostaining of intercellular junctional proteins at cell borders as well as by functional measurements of FITC-dextran flux and TER across epithelial monolayers. This is supported by previous reports from the literature (1, 14, 26). Moreover, TER 14 days postconfluence was $92 \pm 0.2\ \Omega \cdot cm^2$, which was comparable to colon mucosal resistance measured in adult rats ex vivo (1). In summary, our data confirm that Caco2 monolayers can be used as suitable model for investigations of epithelial intestinal barrier functions in vitro.

Dsg2 antibodies can be used as a valid approach to study the role of Dsg2-mediated adhesion in intestinal epithelium. Although desmosomes are known to create strong intercellular associations that are important for the integrity of stratified epithelia such as the epidermis (7, 27), their function in the intestinal epithelium is poorly understood (13). This can in part be explained by the fact that generation of specific knockout mice to address this question was not possible because the specific gene deletion of Dsg2 resulted in early embryonic lethality (5). To get novel insights into the role of Dsg2 for the

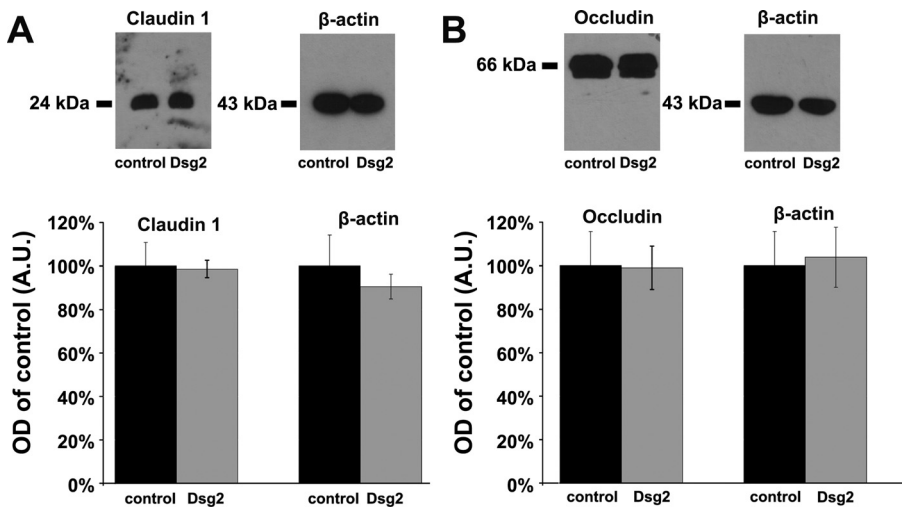

Fig. 9. Total protein amounts of claudin 1 and occludin were unaltered after Dsg2 ED treatment. Representative Western blots of claudin 1 (*A*) and occludin (*B*) are shown under control conditions and after treatment with Dsg2 ED monoclonal for 24 h. Equal protein loading was verified by reprobing the membranes with β-actin antibodies. Results from all experiments ($n = 6$ for each condition) were quantified and are shown in bar graphs below the Western blots. Neither change in total protein amounts of claudin 1 nor of occludin was found in these experiments. OD, optical density.

maintenance of the intestinal epithelial barrier, we hypothesized that application of specific antibodies directed against the Dsg2 ED would disrupt interaction of Dsg2 molecules. This idea derived from studies on the pathogenesis of the skin-blistering autoimmune disease pemphigus vulgaris. Here, we demonstrated that antibodies directed against Dsg3 ED are able to disrupt homophilic interactions between desmogleins (9). This mechanism was shown to contribute to cell dissociation because specific tandem-peptides that bind to Dsg3 ED were effective to stabilize Dsg3 interaction and thereby partially prevented cell dissociation (8). On the basis of our AFM data and the enterocyte-dissociation assay in our present study, it can be concluded that the approach of applying specific antibodies against Dsg2 can be used to study the role of Dsg2-mediated adhesion in Caco2 monolayers. Because we obtained similar results when we applied a commercially available monoclonal antibody directed against Dsg2 ED, it can be concluded that the observed effects in Caco monolayers were specifically induced by interfering with Dsg2 interaction. This is supported by the fact that absorption of the Dsg2 antibodies abolished loss of TER and tight junction disruption and significantly reduced cell dissociation. Similarly no alterations of TER were found when Dsg2 antibody directed against the IC was applied to Caco2 monolayers. Furthermore, this indicates that side effects of the antibody dilutions such as serum proteins in Dsg2 ED polyclonal are negligible. The observation that absorption of Dsg2 antibodies, which was not complete as revealed by Western blotting, was effective to completely block tight junction disruption and loss of TER but only partially inhibited the effect on cell cohesion in dissociation assays indicates that Dsg2-mediated binding was compromised under these conditions but did not cause barrier breakdown unless substantial mechanical force was applied, such as in the dispase-based dissociation assay.

Dsg2-mediated adhesion is required for maintenance of intestinal epithelial barrier integrity. It is well established that mechanical intercellular adhesion of epithelial cells is maintained by homophilic interactions of E-cadherin that form adherens junctions. Moreover, it is known that intact adherens junctions are a prerequisite for integrity of tight junctions (23, 25). This is also supported by our data, where E-cadherin immunostaining at cell borders was clearly present when Caco2 cells became confluent, whereas mature tight junctions were evident considerably later, i.e., after 14 days postconfluence. The present data demonstrate that Dsg2-mediated adhesion is required for maintenance of intestinal epithelial barrier functions because application of Dsg2-specific antibodies directed against Dsg2 ED significantly reduced epithelial intestinal barrier functions as revealed by TER measurements. Moreover, we demonstrated that Dsg2-mediated adhesion is indeed involved in stabilization of intercellular adhesion because Dsg2 ED antibody significantly increased cell dissociation of Caco monolayers. However, because the effect of EGTA in the cell dissociation assay was much more pronounced compared with Dsg2 ED antibody treatment, it can be concluded that other Ca^{2+}-dependent adhesion proteins such as Dsc2 and E-cadherin also contribute to the adhesive forces between intestinal epithelial cells.

In our study it appeared that Dsg2 ED antibody was effective to reduce barrier properties of epithelial monolayers because it led to rupture of tight junctions as revealed by immunostaining. Therefore, although it has been reported that desmosomes form when tight junction formation is complete (4), our data indicate that tight junction integrity requires intact desmosomal adhesion. The relatively moderate loss of TER by ~20% of baseline may be explained by the fact that, not in all areas of the monolayers, tight junctions were completely ruptured. The mechanism by which Dsg2 ED antibodies caused rupture of tight junctions is unclear at present. Moreover, it is not known how the antibody gains access to Dsg2 molecules to induce its effects. Most likely, direct inhibition of Dsg2 transinteraction within desmosomes is achieved by permeation of some amount of antibody across the tight junctions. Afterward, it is conceivable that direct inhibition of Dsg2 transinteraction is sufficient to interfere with maintenance of tight junctions and to further enhance antibody permeation. Alternatively, it is also possible that the antibody binds to single extradesmosomal Dsg2 molecules expressed on the apical cell surface and thereby induces signaling events leading to tight junction disruption. Indeed, it has been reported that desmogleins are involved in mediating signaling events that may significantly affect a large number of cellular functions (2, 15, 27). In support of this notion, Nava and colleagues (16) recently demonstrated that Dsg2 can act as a regulator of apoptosis in intestinal epithelium in response to an inflammatory mediator such as interferon-γ. Moreover, in the field of pemphigus research, it is meanwhile well established that extracellular binding of desmoglein antibodies to desmogleins on the cell surface can trigger intracellular signaling pathways, which indirectly result in loss of desmoglein-mediated binding (12, 27, 28). Therefore, on the basis of the observation that impaired Dsg2-mediated adhesion led to reduced tight junction staining and barrier functions, it can be hypothesized that binding of Dsg2 antibodies to Dsg2 on Caco2 cell surface may interfere with signaling events that are required for maintenance of tight junction integrity. Future studies will be required to specifically prove this hypothesis.

In summary our data demonstrate for the first time that Dsg2-mediated adhesion is required to maintain intestinal epithelial barrier properties by contributing to intercellular adhesion of enterocytes, which in turn is required for maintenance of tight junction integrity.

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DISCLOSURES

The authors declare that they have no conflicts of interest.

REFERENCES

1. **Artursson P, Ungell AL, Löfroth JE.** Selective paracellular permeability in two models of intestinal absorption: cultured monolayers of human intestinal epithelial cells and rat intestinal segments. *Pharm Res* 10: 1123–1129, 1993.
2. **Berkowitz P, Chua M, Liu Z, Diaz LA, Rubenstein DS.** Autoantibodies in the autoimmune disease pemphigus foliaceus induce blistering via p38 mitogen-activated protein kinase-dependent signaling in the skin. *Am J Pathol* 173: 1628–1636, 2008.

3. **Caldelari R, de Bruin A, Baumann D, Suter MM, Bierkamp C, Balmer V, Muller E.** A central role for the armadillo protein plakoglobin in the autoimmune disease pemphigus vulgaris. *J Cell Biol* 153: 823–834, 2001.
4. **Collins JE, Fleming TP.** Epithelial differentiation in the mouse preimplantation embryo: making adhesive cell contacts for the first time. *Trends Biochem Sci* 20: 307–312, 1995.
5. **Eshkind L, Tian Q, Schmidt A, Franke WW, Windoffer R, Leube RE.** Loss of desmoglein 2 suggests essential functions for early embryonic development and proliferation of embryonal stem cells. *Eur J Cell Biol* 81: 592–598, 2002.
6. **Farquhar MG, Palade GE.** Junctional complexes in various epithelia. *J Cell Biol* 17: 375–412, 1963.
7. **Getsios S, Huen AC, Green KJ.** Working out the strength and flexibility of desmosomes. *Nat Rev Mol Cell Biol* 5: 271–281, 2004.
8. **Heupel WM, Muller T, Efthymiadis A, Schmidt E, Drenckhahn D, Waschke J.** Peptides targeting the desmoglein 3 adhesive interface prevent autoantibody-induced acantholysis in pemphigus. *J Biol Chem* 284: 8589–8595, 2009.
9. **Heupel WM, Zillikens D, Drenckhahn D, Waschke J.** Pemphigus vulgaris IgG directly inhibit desmoglein 3-mediated transinteraction. *J Immunol* 181: 1825–1834, 2008.
10. **Heupel WM, Baumgartner W, Laymann B, Drenckhahn D, Golenhofen N.** Different Ca2+ affinities and functional implications of the two synaptic adhesion molecules cadherin-11 and N-cadherin. *Mol Cell Neurosci* 37: 548–558, 2008.
11. **Jörgensen VL, Nielsen SL, Espersen K, Perner A.** Increased colorectal permeability in patients with severe sepsis and septic shock. *Intensive Care Med* 32: 1790–1796, 2006.
12. **Kitajima Y.** Mechanisms of desmosome assembly and disassembly. *Clin Exp Dermatol* 27: 684–690, 2002.
13. **Laukoetter MG, Nava P, Nusrat A.** Role of the intestinal barrier in inflammatory bowel disease. *World J Gastroenterol* 14: 401–407, 2008.
14. **Matsumoto H, Erickson RH, Gum JR, Yoshioka M, Gum E, Kim YS.** Biosynthesis of alkaline phosphatase during differentiation of the human colon cancer cell line Caco-2. *Gastroenterology* 98: 1199–1207, 1990.
15. **Muller EJ, Williamson L, Kolly C, Suter MM.** Outside-in signaling through integrins and cadherins: a central mechanism to control epidermal growth and differentiation? *J Invest Dermatol* 128: 501–516, 2008.
16. **Nava P, Laukoetter MG, Hopkins AM, Laur O, Gerner-Smidt K, Green KJ, Parkos CA, Nusrat A.** Desmoglein-2: a novel regulator of apoptosis in the intestinal epithelium. *Mol Biol Cell* 18: 4565–4578, 2007.
17. **Rowlands BJ, Soong CV, Gardiner KR.** The gastrointestinal tract as a barrier in sepsis. *Br Med Bull* 55: 196–211, 1999.
18. **Sambuy Y, De Angelis I, Ranaldi G, Scarino ML, Stammati A, Zucco F.** The Caco-2 cell line as a model of the intestinal barrier: influence of cell and culture-related factors on Caco-2 cell functional characteristics. *Cell Biol Toxicol* 21: 1–26, 2005.
19. **Schafer S, Stumpp S, Franke WW.** Immunological identification and characterization of the desmosomal cadherin Dsg2 in coupled and uncoupled epithelial cells and in human tissues. *Differentiation* 60: 99–108, 1996.
20. **Schlegel N, Baumer Y, Drenckhahn D, Waschke J.** Lipopolysaccharide-induced endothelial barrier breakdown is cAMP dependent in vivo and in vitro. *Crit Care Med* 37: 1735–1743, 2009.
21. **Schlegel N, Burger S, Golenhofen N, Walter U, Drenckhahn D, Waschke J.** The role of VASP in regulation of cAMP- and Rac 1-mediated endothelial barrier stabilization. *Am J Physiol Cell Physiol* 294: C178–C188, 2008.
22. **Schlegel N, Waschke J.** VASP is involved in cAMP-mediated Rac 1 activation in microvascular endothelial cells. *Am J Physiol Cell Physiol* 296: C453–C462, 2009.
23. **Sousa S, Lecuit M, Cossart P.** Microbial strategies to target, cross or disrupt epithelia. *Curr Opin Cell Biol* 17: 489–498, 2005.
24. **Swank GM, Deitch EA.** Role of the gut in multiple organ failure: bacterial translocation and permeability changes. *World J Surg* 20: 411–417, 1996.
25. **Taddei A, Giampietro C, Conti A, Orsenigo F, Breviario F, Pirazzoli V, Potente M, Daly C, Dimmeler S, Dejana E.** Endothelial adherens junctions control tight junctions by VE-cadherin-mediated upregulation of claudin-5. *Nat Cell Biol* 10: 923–934, 2008.
26. **Wang Q, Guo XL, Wells-Byrum D, Noel G, Pritts TA, Ogle CK.** Cytokine-induced epithelial permeability changes are regulated by the activation of the p38 mitogen-activated protein kinase pathway in cultured Caco-2 cells. *Shock* 29: 531–537, 2008.
27. **Waschke J.** The desmosome and pemphigus. *Histochem Cell Biol* 130: 21–54, 2008.
28. **Waschke J, Spindler V, Bruggeman P, Zillikens D, Schmidt G, Drenckhahn D.** Inhibition of Rho A activity causes pemphigus skin blistering. *J Cell Biol* 175: 721–727, 2006.