Tightening of Endothelial Cell Contacts: A Physiologic Response to Cocultures with Smooth-Muscle-Like 10T1/2 Cells

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Tightening of endothelial cell-to-cell contacts is an important event at the end of angiogenesis in order to achieve controlled transfer of solutes between the blood stream and solid tissues. We found that tightening of endothelial cell-to-cell contacts and the formation of a permeability barrier can be induced in vitro by dibutyryl cAMP and hydrocortisone. This process is accompanied by increased junctional localization and cytoskeletal association of the adherens junctional plakoglobin and the tight junction associated proteins ZO-1, ZO-2, and occludin. Based on these findings, we proceeded to investigate whether smooth-muscle-like mesenchymal cells would influence endothelial junctional differentiation. For this purpose, human umbilical cord vein endothelial cells and murine smooth-muscle-like 10T1/2 cells were cocultivated and compared with their respective monocultures. Immunofluorescence on cells and Western blot analyses were performed for marker proteins of adherens and tight junctions. Functional permeability assays were performed for the tracer molecule biotin-dextran. The results indicated that 10T1/2 cells induced the tightening of endothelial cell-to-cell contacts. Plakoglobin, ZO-1, ZO-2, and occludin showed increased junctional localization when 10T1/2 cells were present. Cocultures also displayed a significantly higher permeability barrier for the tracer molecule biotin-dextran. In conclusion, mural cells such as smooth muscle cells and pericytes may be important for stabilizing endothelial cell-to-cell contacts and may influence vessel-type specific differences of the endothelial phenotype. Key words: adherens junction/angiogenesis/cell culture/differentiation/tight junction. J Invest Dermatol 119:143–153, 2002

Angiogenesis is the formation of new capillaries from pre-existing blood vessels (Risau, 1995; Wilting and Christ, 1996). For angiogenesis to proceed, the endothelial cells first have to loosen their cell-to-cell contacts, degrade the underlying basement membrane, migrate into the surrounding tissue, and proliferate, after which the endothelial cells realign and form a new capillary sprout. In order for the endothelium to form a selective barrier between the blood stream and solid tissues, vascular maturation requires the tightening of endothelial cell-to-cell contacts and the down-regulation of endothelial proliferation, the deposition of a basal lamina to which the endothelium tightly adheres, and the recruitment of supporting cells to the vessel walls such as pericytes and smooth muscle cells (Grant et al, 1991; Lampugnani and Dejana, 1997; Risau, 1997; Balda and Matter, 1998; Hirschi et al, 1998). Endothelial barrier formation is achieved by the establishment of specific, but functionally still less well understood, adhering and tight junctions (Schnittler, 1998; Vestweber, 2000).

Epithelial adhering junctions are well characterized by an electron-dense cytoplasmic plaque, in which transmembrane glycoproteins of the cadherin family connect via specific cytoplasmic plaque proteins of the armadillo family to the cytoskeleton (Farquhar and Palade, 1963; Schmidt et al, 1994; Takeichi, 1995). By their junctional composition and the type of filament anchored, two basic types of adhering junctions can be distinguished in epithelia: (i) adherens junctions in which the classical type I cadherins (E-, N-, P-, R-cadherin) link via α-catenin, β-catenin, and plakoglobin to the actin microfilaments; and (ii) desmosomes in which the desmosomal cadherins (desmogleins and desmocollins) connect through desmoplakin, plakophilins, and plakoglobin to the intermediate filaments (Schmidt et al, 1994; Takeichi, 1995). Adherens junctions are known to form lateral clusters that seem to be mediated by p120ctn, another member of the armadillo family. The initial cell-to-cell contacts are mediated by the classical cadherins and adherens junctions, thus leading to further tightening through desmosomes and tight junctions (Adams and Nelson, 1998; Watabe-Uchida et al, 1998). These tight junctions are characterized by the transmembrane proteins occludin and the growing family of claudins (Balda and Matter, 1998; Furuse et al, 1998). The tight junctional cytoplasmic complexes include the proteins ZO-1, ZO-2, ZO-3, cingulin, 7H6, and symplekin (Keon et al, 1996), which mediate the linkage to the actin cytoskeleton.

In contrast to the epithelium, endothelial junctional complexes do not show a clear spatial organization and morphologic differentiation into adhering junctions and apical tight junctions (Franke et al, 1988; Schnittler, 1998; Vestweber, 2000). Instead, both junctional structures seem to be present along the complete lateral aspect of the endothelial contacting zones. Furthermore,
despite the reported presence of desmosomal plaque proteins desmoplakin and plakoglobin (Schmelz et al, 1994; Kowalczyk et al, 1998), endothelial cells do not have structures that resemble classical desmosomes. In the endothelium, “adherens junctions” seem to connect to both the actin microfilaments and the intermediate filaments of the vimentin type (Kowalczyk et al, 1998). The plaque proteins α-catenin, β-catenin, plakoglobin, and desmoplakin link the cytoskeleton to VE-cadherin, the only classical cadherin so far reliably identified in endothelial cell junctions. In contrast, N-cadherin, although present in endothelial cells, is thought to be distributed evenly over the plasma membrane without restriction to junctional complexes (Salomon et al, 1992).

To gain insight into the functional organization of the endothelial cell-to-cell contacts, we utilized an in vitro model for the study of endothelial junctions. In this model, we focused on the basis of this model that the cultivation of endothelial cells in the presence of dibutyryl cAMP (B2) and hydrocortisone (HC) induced a phenotype of differentiated endothelial cell. The characteristics of this differentiated endothelial phenotype were the deposition of a basement membrane type of matrix, reduced cell proliferation, tightening of the endothelial adherens junctions, and an increased association of the plaque proteins plakoglobin and p120ctn with the actin microfilament system (Kräling et al, 1999; Koch et al, 2000). In this study, we analyzed the organization of the endothelial tight junctions and the establishment of an endothelial permeability barrier. To find evidence for a physiologic role of the endothelial differentiation in the presence of B2 and HC, we examined the organization of the plaque proteins α-catenin, β-catenin, and plakoglobin in the following primary antibodies were used in this study: mouse antihuman VE-cadherin (clone TEA1.31, Immunotech, Marseille, France; and clone BV9, a kind gift from Dr. Elisabetta Dejana, Mario Negri Institute, Mailand, Italy) (Leach et al, 1993); mouse antihuman N-cadherin (clone 32, Transduction Laboratories, Lexington, MA) and mouse anti-N-cadherin (clone 3B9, Zyymed, San Francisco, CA); mouse antihuman p120 (clone CH103, Transduction Laboratories) and rabbit antisera against α-catenin (Sigma, St. Louis, MO); mouse antihuman β-catenin (clone 14, Transduction Laboratories) and polyclonal rabbit anti-β-catenin (Sigma); mouse antihuman plakoglobin (clone I1E4, a gift from Margaret Wheelock, Toronto, Canada); mouse antihuman p120 (clone 20, Transduction Laboratories); rabbit antihuman cadherin (clone 2F6C2, Sigma); rabbit anti-ZO-1, rabbit anti-ZO-2, rabbit anti-caduculin, rabbit antihuman claudin-1 antisera (all from Zymed); rabbit antihuman claudin-5/6 serum, a kind gift from Dr. Shochiro Tsuchita, Kyoto, Japan (Morita et al, 1999); mouse antihuman vimentin (clone 3B4, Progen; Heid et al, 1988), rabbit antihuman von Willebrand factor (Erik Patts, Denmark).

The following secondary antibodies were used: Cy-2 and Cy-3 conjugated goat antimouse, rabbit, and guinea pig antibodies obtained from Dianova; fluorescein-isothiocyanate– and Texas red-conjugated goat antirabbit antibodies (Dianova); Alexa 488 and Alexa 594 conjugated goat antimouse, rabbit, and guinea pig antibodies (Molecular Probes, and horseradish peroxidase–HRP conjugated horse antimouse IgG (Vector Laboratories, Burlingame, CA); HRP-conjugated goat antirabbit IgG (Vector Laboratories). MATERIALS AND METHODS

Antibodies used in this study

The following primary antibodies were used in this study: mouse antihuman VE-cadherin (clone TEA1.31, Immunotech, Marseille, France; and clone BV9, a kind gift from Dr. Elisabetta Dejana, Mario Negri Institute, Mailand, Italy) (Leach et al, 1993); mouse antihuman N-cadherin (clone 32, Transduction Laboratories, Lexington, MA) and mouse anti-N-cadherin (clone 3B9, Zyymed, San Francisco, CA); mouse antihuman p120 (clone CH103, Transduction Laboratories) and rabbit antisera against α-catenin (Sigma, St. Louis, MO); mouse antihuman β-catenin (clone 14, Transduction Laboratories) and polyclonal rabbit anti-β-catenin (Sigma); mouse antihuman plakoglobin (clone I1E4, a gift from Margaret Wheelock, Toronto, Canada); mouse antihuman p120 (clone 20, Transduction Laboratories); rabbit antihuman cadherin (clone 2F6C2, Sigma); rabbit anti-ZO-1, rabbit anti-ZO-2, rabbit anticaduculin, rabbit antihuman claudin-1 antisera (all from Zymed); rabbit antihuman claudin-5/6 serum, a kind gift from Dr. Shochiro Tsuchita, Kyoto, Japan (Morita et al, 1999); mouse antihuman vimentin (clone 3B4, Progen; Heid et al, 1988), rabbit antihuman von Willebrand factor (Erik Patts, Denmark).

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Cultures were maintained at 5% CO₂ and 37°C in a humidified incubator and analyzed after 3 d. Cells were washed with HEPES wash buffer (10 mM HEPES, pH 7.4, 135 mM NaCl, 5 mM KCl, 1 mM MgSO₄, and 1.8 mM CaCl₂), directly scraped into 0.5 ml 2X SDS sample buffer, and analyzed by SDS-PAGE and Western blotting as described above. For microscopic analysis, the glass coverslips were removed and processed as described for immunofluorescence staining. All experiments were repeated at least twice.

**Permeability assay on endothelial cells and cocultures**

Endothelial cell monolayers were plated on top of gelatinized Transwell chambers for 24-well plates (0.4 µm pores) (Becton Dickinson) at densities of 1 × 10⁵ cells per well. When they had reached confluence at day 5, one set of cells was treated with Bt2 and HC and one set was maintained in basal medium for an additional 4 d at which point the permeability assays were performed. All experiments were repeated at least twice.

Cocultures of 10T1/2 cells with endothelial cells. Gelatinized filters of Transwell chambers for 24-well plates (Becton Dickinson) were seeded with 10T1/2 and endothelial cells as described above except that 5 × 10⁵ 10T1/2 cells in endothelial growth medium were plated onto the bottom side of the filter membranes and 1 × 10⁵ endothelial cells in endothelial growth medium were plated onto the upper side of the filter membranes. Control cultures were established with either endothelial cells alone or 10T1/2 cells alone in comparable cell numbers (5 × 10⁵ cells onto the bottom side and 1 × 10⁵ cells onto the top side of the filter membrane). The cultures were maintained at 5% CO₂ and 37°C in a humidified incubator with fresh medium every third day for a total of 8 d when the permeability assays were performed.

**Permeability assay**

The tracer molecule biotin-dextran (Sigma) (≈70 kDa) was added to the top compartment of the Transwells at a concentration of 200 µg per ml. The permeability of the cell layers for this tracer molecule was measured by collecting aliquots from the bottom compartments. For this purpose, at each indicated time point, the solution from the bottom compartment was aspirated and replaced by 0.5 ml HEPES-buffered saline (10 mM HEPES, pH 7.4, 135 mM NaCl, 5 mM KCl, 1 mM MgSO₄, and 1.8 mM CaCl₂). Duplicate aliquots (2 × 35 µl) for sample detection were removed after 10 min. Sample detection occurred by regular enzyme-linked immunosorbert assay (ELISA) methods (Kraling and Bischoff, 1998) on streptavidin-coated 96-well microtiter plates (MicroCoat, Germany) with HRP-conjugated mouse antibiotin antibody (Sigma). Tetramethylbenzidin (Sigma) was used as a substrate for HRP and the color reaction was stopped with 2 N H₂SO₄ after 15 min. Color intensities were measured at 450 nm using a Titertek Multiscan plate reader. The background was calculated as the mean value from measuring pure endothelial growth medium, pure FBS, and pure biotin (200 µg per ml) in endothelial growth medium. The mean background was subtracted from the raw data and the mean value and standard deviation for each data point were calculated. Statistical analysis on the data sets was performed by Student’s t test.

**RESULTS**

**Adherens junctions in the differentiated endothelial phenotype**

Previously, the induction of a differentiated endothelial phenotype was described after treatment of endothelial cell cultures with Bt2 and HC (Kraling et al., 1999; Koch et al., 2000). One hallmark of this differentiated phenotype was the tightening of endothelial cell-to-cell contacts after addition of Bt2 and HC. In contrast, endothelial cells in basal conditions showed small gaps in the contacting zones between individual cells even after reaching confluence (Koch et al., 2000). The mechanisms for closing the endothelial cell-to-cell contacts could be the elongation of interdigitations (not shown) or the formation of increased numbers of interdigitations between neighboring endothelial cells that could be observed after treatment with Bt2 and HC (Fig 1A’). Figure 1. Increased interdigitations between neighboring HDMEC after treatment with Bt2 and HC. Electron microscopy on representative cross-sections of confluent HDMEC after treatment for 3 d with basal conditions or Bt2 and HC revealed that Bt2- and HC-treated endothelial cells showed stronger interdigitations (*) between neighboring endothelial cells (A’) than those in basal conditions (A). Scale bar: 600 nm.

Negative immunofluorescence staining in the absence of primary antibodies. In basal conditions, N-cadherin (B) and VE-cadherin (C) were present at endothelial cell-to-cell contacts. The respective pattern of these components appeared focal and discontinuous. After treatment with Bt2 and HC, the junctional localization of N-cadherin almost disappeared (B’) whereas VE-cadherin (C) changed to continuous lines with prominent signals. Scale bar: 50 µm.

Figure 2. Differential localization of constituents of adherens junctions during endothelial maturation in vitro. Immunofluorescence staining was performed on confluent HUVEC after 3 d in basal conditions (A–C) or treatment with Bt2 and HC (A’–C’) for N-cadherin (B, B’) and VE-cadherin (C, C’). Panels A and A’ show negative immunofluorescence staining in the absence of primary antibodies. In basal conditions, N-cadherin (B) and VE-cadherin (C’) were present in endothelial cell-to-cell contacts. The respective pattern of these components appeared focal and discontinuous. After treatment with Bt2 and HC, the junctional localization of N-cadherin almost disappeared (B’) whereas VE-cadherin (C’) changed to continuous lines with prominent signals. Scale bar: 50 µm.
after immunofluorescence staining at the endothelial cell-to-cell contacts in basal growth conditions (Fig 2B), became barely detectable after treatment with Bt2 and HC (Fig 2B'). The observed downregulation of N-cadherin after treatment with Bt2 and HC was further confirmed by Western blot analysis (Fig 3a). Treatment with Bt2 and HC led to decreased protein levels of N-cadherin and lessened the association of this cell adhesion molecule with the cytoskeleton by 2-fold as shown by densitometry (data not shown).

**Localization and cytoskeletal association of tight junction associated constituents after treatment with Bt2 and HC** Confluent HDMEC and HUVEC were treated with Bt2 and HC for 3 d whereas controls were maintained in basal medium. Claudin-1 and claudin-5 were present in scattered endothelial cells (Fig 4A). Junctional labeling for ZO-1 and ZO-2 was focal and discontinuous (Fig 4B, D, E). Ocludin was barely present at the cell-to-cell contacts in basal conditions (Fig 4C). In contrast, Bt2- and HC-treated endothelial cell cultures displayed regularly delineated cell-to-cell contacts. The junctions showed prominent staining for occludin, ZO-1, and ZO-2, which were visible as continuous lines (Fig 4B'-E'). Claudin-1 and claudin-5 were increasingly found at the sites of cell contacts with a heterogeneous distribution within the monolayer (Fig 4A').

The increased localization of the tight junctional proteins at the endothelial cell-to-cell contacts after Bt2 and HC treatment was accompanied by an increased association of these components with the cytoskeleton, which could be clearly demonstrated after differential extraction of endothelial monolayers with digitonin and detergents (Fig 3a). First, the soluble components of the cytoplasm were released with 50 μg per ml digitonin. The remaining pellets were extracted with buffer A (25 mM Tris–HCl, pH 7.4, 2% Nonidet P-40) or buffer B (PBS with 1% Triton X–100, 0.5% DOC, 0.005% SDS, and 1 mM EDTA) to release those components that were weakly associated with the cytoskeleton. The tightly associated constituents remained in the residual pellets. The increased association of ZO-1, ZO-2, and occludin with the cytoskeleton was most obvious when comparing the residual pellets after the basal condition (0) with those after Bt2 and HC treatment (+) (Fig 3a, compare lane 7 with lane 8 or lane 9 with lane 10). The increased association of ZO-1 with the cytoskeleton was observed after extraction with buffer A whereas that of ZO-2 and occludin was demonstrated after extraction with buffers A and B. Densitometry revealed that the levels of ZO-1 and ZO-2 in the insoluble fraction increased by a factor of 2 and occludin by a factor of 3 (data not shown).

Additionally, the observed tightening of the endothelial cell-to-cell contacts had a functional effect on HDMEC and HUVEC as the formation of a significantly increased permeability barrier to the tracer molecule biotin-dextran could be observed after Bt2 and HC treatment (Fig 3b) (p < 0.0001). The permeability assays furthermore revealed that HUVEC had an intrinsically higher capability for barrier formation than HDMEC. This may reflect vessel-type-specific differences between these endothelial cells due to their origin from dermal microvessels (HDMEC) in comparison to large veins (HUVEC).

**Endothelial junctional differentiation in the presence of smooth-muscle-cell-like 10T1/2 cells** We established cocultures between HUVEC and 10T1/2 cells in order to

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**Figure 3. Cytoskeletal association of tight junctional constituents in endothelial cells during junctional differentiation and the formation of an increased permeability barrier.** (a) Detergent soluble and insoluble fractions of HUVEC were separated by 8% SDS-PAGE followed by Western blot analysis. Lanes 1, 2, 3, 7, and 9 represent basal conditions; lanes 4, 5, 6, 8, and 10 represent Bt2 and HC treatment. Lanes 1 and 4 show cell lysates with 50 μg per ml digitonin, lanes 2 and 5 extracts with buffer A (25 mM Tris–HCl, pH 7.4, 2% Nonidet P-40), and lanes 3 and 6 extracts with buffer B (PBS with 1% Triton X–100, 0.5% DOC, 0.005% SDS, and 1 mM EDTA). The insoluble pellets after extraction with buffer A are shown in lanes 7 and 8; the insoluble pellets after extraction with buffer B in lanes 9 and 10. Treatment with Bt2 and HC resulted in decreased levels of N-cadherin in the detergent soluble fractions (compare lane 2 with lane 5 and lane 3 with lane 6) as well as in the detergent insoluble fractions (compare lane 7 with lane 8 and lane 9 with lane 10). In contrast, ZO-1, ZO-2, and occludin became more closely associated with the cytoskeleton after Bt2 and HC treatment, which was most obvious after extraction with buffer A (compare lane 7 with lane 8). Gel loading was controlled by vimentin levels. (b) Permeability assays for the tracer molecule biotin-dextran were performed on confluent monolayers of HUVEC and HDMEC in Transwell chambers 3 d after treatment with basal conditions or Bt2 and HC. Biotin-dextran (200 μg per ml) was added to the top compartment and aliquots were taken from the bottom compartment after 40 min to be analyzed by regular ELISA on streptavidin-coated microtiter plates. Bt2 and HC significantly increased the permeability barrier in both endothelial cell lines (gray bars) compared to basal conditions (white bars) (p < 0.0001). HUVEC showed an intrinsically higher ability for barrier formation than HDMEC in basal conditions as well as after in vitro differentiation.
investigate whether the presence of smooth-muscle-like cells would influence endothelial junctional differentiation. First, we analyzed the production of junctional components in monocultures of endothelial and 10T1/2 cells in comparison to their mixed coculture by Western blot analysis (Fig 5a, b). Cocultures were established of HUVEC to 10T1/2 cells at a ratio of 2:1.
Monocultures of either HUVEC and 10T1/2 cells had cell numbers that were equal to the total cell count of the cocultures. Gel loading was normalized to total cell numbers and was controlled by Coomassie blue staining of PVDF membranes after blotting or of polyacrylamide gels after SDS-PAGE.

HUVEC were positive for plakoglobin and the endothelial marker protein VE-cadherin, neither of which were detected in 10T1/2 monocultures (Fig 5a). In contrast, 10T1/2 cells were positive for α-SMC-actin, which verified their smooth muscle cell phenotype (Fig 5a). In addition, 10T1/2 cell monocultures produced high levels of N-cadherin (Fig 5a). Other components of adherens junctional complexes, α-catenin, β-catenin, and p120, were produced in both HUVEC and 10T1/2 monocultures. With respect to tight junctional proteins in monocultures, ZO-1, ZO-2, and occludin were almost absent from 10T1/2 cells but clearly present in HUVEC (Fig 5b). The reduction in protein levels observed in cocultures may result from the reduced cell numbers of HUVEC or 10T1/2 cells in coculture in comparison to monocultures. As plakoglobin, ZO-1, and occludin were basically absent from 10T1/2 cells, these proteins were utilized to further analyze the junctional differentiation of HUVEC in the presence of the smooth-muscle-like 10T1/2 cells.

Immunofluorescence studies were performed in parallel on HUVEC in monocultures and in cocultures with 10T1/2 cells (Figs 6–8). Both cultures were fixed and analyzed at subconfluence 3 d after establishing the cultures. Otherwise, 10T1/2 cells would have overgrown the endothelial cells in cocultures. HUVEC monocultures showed large and extended endothelial cells (Fig 6A–D), VE-cadherin (Fig 6A), ZO-1 (Fig 6C), and ZO-2 (Fig 6D) could be detected at sites of endothelial cell-to-cell contact. The pattern of the staining was focal and discontinuous. Plakoglobin was rarely detected in HUVEC during monoculture (Fig 6B). In contrast, in the presence of smooth-muscle-like 10T1/2 cells, HUVEC were found in compacted islands that were surrounded by 10T1/2 cells (arrows in Fig 6; asterisks in Fig 7). The islands of HUVEC were identified by labeling for VE-cadherin (Fig 6A') and were characterized by compacted endothelial cells. The endothelial cell-to-cell contacts showed prominent and continuous labeling for VE-cadherin (Fig 6A'), plakoglobin (Fig 6B'), ZO-1 (Fig 6C'), and ZO-2 (Fig 6D'). In addition, occludin was almost absent from HUVEC in monoculture (Fig 7A) but was detected at cell-to-cell contacts of HUVEC in cocultures (arrows in Fig 7B) where it codistributed with VE-cadherin (arrows in Fig 7C).

Endothelial monocultures displayed junctional codistribution of VE- and N-cadherin (Fig 8A–C; arrowheads in Fig 8A). Focally, N-cadherin appeared to be even more prominent than the VE-cadherin (arrows in Fig 8A). After cocultivating HUVEC with 10T1/2 cells, however, VE-cadherin could be detected at the sites of endothelial cell-to-cell contacts whereas N-cadherin was absent (arrowheads in Fig 8D). In these cultures, N-cadherin was merely associated with contacting 10T1/2 cells (arrows in Fig 8D). These results indicate that N-cadherin became downregulated at the endothelial cell-to-cell contacts after cocultivation with mesenchymal 10T1/2 cells.

To functionally analyze the effect of smooth-muscle-like mesenchymal cells on the tightness of the endothelium, we measured the transendothelial flux of the small tracer molecule biotin-dextran in HUVEC monocultures and cocultures with 10T1/2 cells. We found that these cocultures showed a significantly increased permeability barrier compared with HUVEC in monocultures (p < 0.01) (Fig 9). In summary, the presence of smooth-muscle-like 10T1/2 cells induced junctional differentiation in endothelial cells, which was phenotypically and functionally similar to the in vitro differentiation after Bt2 and HC treatment. Treatment with Bt2 and HC or coculture with smooth-muscle-like cells induced a differentiated endothelial phenotype that was characterized by enhanced junc-
tional localization and tighter cytoskeletal association of plakoglobin, ZO-1, and occludin, thus functionally leading to an increased barrier formation.

DISCUSSION

We have established an in vitro model for endothelial differentiation, which we have exploited to examine the junctional composition of endothelial cells in the last stages of angiogenesis. The purpose of this study was to analyze the effect of different growth conditions on the tightening of endothelial cell-to-cell contacts and barrier formation. First, we investigated endothelial cell-to-cell contacts, focal signals for VE-cadherin (A), ZO-1 (C), and ZO-2 (D) could be observed whereas plakoglobin (B) could barely be detected. In cocultures with 10T1/2 cells, the HUVEC became visible after staining for VE-cadherin as islands of compacted cells with dense and uninterrupted cell-to-cell contacts (A'). In contrast to the monocultures, the contacting zones between endothelial cell islands in coculture displayed prominent and continuous signals for plakoglobin (B'), ZO-1 (C'), and ZO-2 (D'). The arrows point at 10T1/2 cells in cocultures. Scale bar: 50 μm.

Figure 6. Compacted endothelial cell islands in cocultures with 10T1/2 cells. Corresponding monocultures of HUVEC (A–D) and cocultures between HUVEC and 10T1/2 cells (A’–D’) were fixed in methanol and labeled for VE-cadherin (A, A’), plakoglobin (B, B’), ZO-1 (C, C’), and ZO-2 (D, D’). In endothelial monocultures, the endothelial cell bodies were large and spread out, and gaps were apparent between the endothelial cells. At the sites of endothelial cell-to-cell contacts, focal signals for VE-cadherin (A), ZO-1 (C), and ZO-2 (D) could be observed whereas plakoglobin (B) could barely be detected. In cocultures with 10T1/2 cells, the HUVEC became visible after staining for VE-cadherin as islands of compacted cells with dense and uninterrupted cell-to-cell contacts (A’). In contrast to the monocultures, the contacting zones between endothelial cell islands in coculture displayed prominent and continuous signals for plakoglobin (B’), ZO-1 (C’), and ZO-2 (D’). The arrows point at 10T1/2 cells in cocultures. Scale bar: 50 μm.

Figure 7. Occludin displayed a junctional localization in compacted endothelial cell islands after coculture with 10T1/2 cells. Immunofluorescence labeling of HUVEC in monocultures showed a diffuse staining for occludin (A), which was not detectable at sites of cell-to-cell contact. In cocultures of HUVEC and 10T1/2 cells, however, occludin could be observed at endothelial cell-to-cell junctions (arrows in B) where it codistributed with VE-cadherin (arrows in C). 10T1/2 cells are designated by asterisks. Scale bar: 50 μm.

vated with bovine aortic endothelial cells, changed to a smooth-muscle-like phenotype expressing marker proteins of smooth muscle cells such as calponin, α-smooth muscle cell actin (α-SMC-actin) and SMC-myosin (Hirschi et al, 1998). Therefore, we obtained this cell line for establishing cocultures between HUVEC and 10T1/2 cells in order to investigate whether the presence of smooth-muscle-like cells would influence endothelial junctional differentiation. We observed that coculture between endothelial
cells and 10T1/2 cells led to a tightening of the endothelial cell-to-cell contacts, which was also confirmed on a functional level by comparing the permeability of cocultures to monocultures for the tracer molecule biotin-dextran. Thus, treatment with Bt2 and HC caused similar changes to the endothelial phenotype as were observed after coculture with 10T1/2 cells.

The majority of transendothelial solute exchange occurs paracellularly at the interendothelial junctions (Shasby et al., 1982; Franke et al., 1988; Alexander et al., 1993; Schnittler, 1998; Vestweber, 2000). Our results indicate that the tightness of the endothelial barrier is mediated through the degree of interdigitation between endothelial cells and the stability of the endothelial junctions. Previous studies have shown that a tight association of junctional molecules with the cytoskeleton is required for the stability of interendothelial adhesion (Lampugnani et al., 1995; Dejana et al., 1999). Many physiologic agents have been shown to modulate the permeability of the endothelial barrier. Treatments that increase endothelial cAMP (isoproterenol, Bt2) produce a decrease in permeability, whereas treatments that increase intracellular calcium increase endothelial permeability (Alexander et al., 1993; Koch et al., 2000).

VE-cadherin is the only cadherin reliably localized to endothelial junctions. It is required for vascular morphogenesis, as can be concluded from VE-cadherin-deficient mouse embryos, which show severe defects of the developing vasculature (Carmeliet et al., 1999; Gory-Faure et al., 1999). In the early phase of contact formation, VE-cadherin assembles cytoplasmic β-catenin at the junction and induces contact to the actin cytoskeleton (Halama et al., 1999); in later stages plakoglobin becomes predominantly associated with the adherens junctional complexes (Dejana, 1996; Schnittler et al., 1997; Schnittler, 1998). Another member of the cadherin family, N-cadherin, was cloned from endothelial cells (Salomon et al., 1992). Although N-cadherin-deficient mouse embryos fail to form blood islands (Radice et al., 1997), a functional role for this cadherin in the endothelium is still missing. N-cadherin is at least transiently required for the reformation of endothelial junctions after cell culture in low calcium (Alexander et al., 1993).
Support for an early involvement of N-cadherin in the process of angiogenesis was also observed during blood–brain barrier maturation. Initially, β-catenin, but not plakoglobin, is codistributed with N-cadherin at the abluminal endothelial membrane at contact sites to perivascular cells. In contrast, plakoglobin is most prominent at the interendothelial junctions where only small amounts of β-catenin are present. In contrast to β-catenin and plakoglobin, N-cadherin is completely lost during later stages of development (Liebner et al, 2000). The presence of VE-cadherin has been shown to exclude N-cadherin from the junctions (Navarro et al, 1998). This is well in agreement with our observation that the induction of a differentiated endothelial phenotype seems to increase the junctional association of VE-cadherin whereas lowering that of N-cadherin. This leads to the assumption that VE-cadherin is responsible for homotypic cell–to-cell contact, whereas N-cadherin may account for adhesion to different cell types, like smooth muscle cells or pericytes (Gilbertson-Beadling and Fisher, 1993). In contrast to this assumption, we did not observe any staining for N-cadherin at the sites of cell contact between HUVEC and 10T1/2 cells. This might be due to the inability of N-cadherin from different species to form “homophilic” interactions. Another explanation could be that the N-cadherin-mediated interaction between endothelial and mural cells is limited to a very short time window.

The endothelial adherens junction is thought to play a role in tight junction formation, which restricts and regulates solute exchange (Lum and Malik, 1994; Anderson and Van Italie, 1995). Therefore, we analyzed tight junction associated proteins in our model of endothelial differentiation. We found that tight junction associated transmembrane proteins, most prominently occludin, and associated plaque proteins (ZO-1, ZO-2) were significantly upregulated after treatment with Bt2 and HC.

Additionally, we observed that the tightening of the endothelial cell-to-cell contacts had a functional effect on HDMEC and HUVEC. The formation of a significantly increased permeability barrier to the tracer molecule biotin–dextran was detected after treatment with Bt2 and HC (p <0.0001). The permeability assays furthermore revealed that HUVEC had an intrinsically higher capability for barrier formation than HDMEC. These results support previous findings by Kevil et al (1998) who could demonstrate vessel-type-specific differences in vitro for venous and arterial endothelial cells, the latter being much less permeable. Here we show that microvascular endothelial cells are more permeable than endothelial cells derived from large veins. These findings indicate that the intrinsic capability of endothelial cells for the formation of a permeability barrier depends on the vessel of origin. The possible mechanisms by which these differences are maintained in cell culture are part of future investigations.

Coculture between endothelial cells and smooth-muscle-like 10T1/2 cells induced a similar differentiated endothelial phenotype to that observed after treatment with Bt2 and HC, namely tightening of the endothelial cell-to-cell contacts and an increased cytoskeletal association of the endothelial junctional proteins plakoglobin, occludin, and ZO-1. Most strikingly, the presence of 10T1/2 cells significantly increased the permeability barrier of the endothelium. We used HUVEC for these cocultures as they are naturally surrounded by smooth muscle cells within the organism.

Both the endothelium and the mural cells are important for the differentiation of mature and patent blood vessels (Hirschi et al, 1999; Nicosia and Villaschi, 1999; Carmeliet, 2000). In our study, we show that smooth-muscle-like cells induce a differentiated endothelial phenotype in vitro. It is known from previous studies that pericytes and smooth muscle cells inhibit endothelial cell migration and proliferation in cell culture (Orlidge and d’Amore, 1987; Sato and Rifkin, 1989). This inhibitory activity is mediated by transforming growth factor β (TGF-β), which becomes activated when smooth muscle cells or pericytes make contact to endothelial cells (Sato and Rifkin, 1989; Sato et al, 1990). Endothelial cells in turn release platelet derived growth factor BB (PDGF-BB), which induces pericytes and smooth muscle cells to migrate towards the endothelium and to make contact (Hirschi et al, 1998, 1999). Furthermore, mesenchymal fibroblastoid 10T1/2 cells differentiate into a smooth muscle cell phenotype when they make contact to endothelial cells, a process that is inhibited by blocking antibodies to TGF-β (Hirschi et al, 1998). Thus, cells of the vessel wall and endothelial cells reciprocally activate mechanisms that induce vascular differentiation. This is further supported by our findings in this study, which suggest that smooth muscle like cells exert a functionally stabilizing effect on endothelial barrier formation.

Further insight into blood vessel maturation and the importance of the interaction between endothelial and mural cells for blood vessel maturation stems from studies on gene-deficient mice. Mice lacking endoglin, which is a TGF-β binding protein on the surface of endothelial cells, are characterized by severe vascular malformations that are embryonic lethal. In these mice, the capillary plexus does not show proper recruitment of a supporting layer of smooth muscle cells (Li et al, 1999). Severe defects in the establishment of a differentiated vascular network are also observed in mice lacking either PDGF-B or the PDGF-receptor-β (Lindahl et al, 1997; Hellstrom et al, 1999). Again, the capillary plexus lacks supporting mural cells. All these studies furthermore present evidence that the lack of supporting cells causes an arrest in endothelial remodeling.

A primitive capillary network is developed in mice with a homozygous deficiency in the tie-2 tyrosine kinase receptor (Sato et al, 1995), in mice with a homozygous deficiency in the tie-2 ligand angiopoietin-1 (ang-1) (Suri et al, 1996), and in transgenic mice overexpressing its negative ligand ang-2 (Maisonpierre et al, 1997). The primitive capillary network, however, does not become remodeled and blood vessel differentiation does not proceed. The endothelium fails both to adhere tightly to the underlying basement membrane and to recruit mural cells, thus rendering these mice embryonic lethal (for review, see also Hanahan, 1997). Interestingly, mice with a homozygous deletion of the ephrin-B2 gene show a similar phenotype to tie-2 or ang-1 null mice (Wang et al, 1998; Adams et al, 1999, 2001). Ephrins are cell surface bound ligands to Eph receptor tyrosine kinases, which are implicated in repulsive axon guidance, cell migration, and

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**Figure 9.** Increased barrier formation of HUVEC in the presence of 10T1/2 cells. Permeability assays of HUVEC and 10T1/2 cells in monoculture and coculture for biotin–dextran in Transwell chambers. HUVEC and 10T1/2 cells were cultured on opposite sides of the Transwell filter membrane. Controls were set up with HUVEC only or 10T1/2 cells only. After 8 d, the permeability of the confluent cell layers was analyzed. Biotin–dextran was added to the top compartment and aliquots were taken from the bottom compartment after 3 h to be analyzed by regular ELISA methods (compare Fig 3b). Cocultures of HUVEC with 10T1/2 cells (black bar) had a significantly reduced permeability for biotin–dextran compared to HUVEC monocolonies (light gray bar) (p <0.01). Monocolonies of 10T1/2 cells (white bar) had the highest permeability for biotin–dextran.
angiogenesis (Klein, 2001). These mice fail to remodel the primary capillary plexus, resulting in defective developmental angiogenesis. Adams et al (1999) presented evidence that ephrin–Eph interactions could be involved in signaling processes between the endothelium and the adjacent mesenchyme. Furthermore, mice with a homozygous deficiency of the tie-1 orphan receptor die shortly after birth due to edema and severe hemorrhaging, which are also caused by aberrant vessel integrity (Sato et al, 1995).

All these findings in gene-deficient mice describe severe and lethal vascular defects due to malfunctioning cell-to-cell signaling between the endothelium and the supporting mural cells. In our study, we describe how smooth-muscle-like cells can induce the phenotypic differentiation of endothelial cells and the formation of an increased endothelial barrier. Our data underscore the importance of mural cells for stabilizing the vascular endothelium. Therefore, this cell culture system may represent a valuable model to analyze mediators of vascular differentiation that are involved in heterotypic signaling between endothelial and mural cells within a controlled cell culture environment, which will be part of our future studies.

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