Regular Article

Effect of cyclic AMP on barrier function of human lymphatic microvascular tubes ☆

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ABSTRACT

This work examines the effect of cyclic AMP (cAMP) on the in vitro barrier function of tubes of human dermal lymphatic microvascular endothelial cells (LECs). Under baseline conditions, the barrier function of LEC tubes was weak, with diffusional permeability coefficients to bovine serum albumin and 10 kDa dextran of $1.4^{+0.9}_{-0.6} \times 10^{-6}$ cm/s and $1.7^{+0.8}_{-0.5} \times 10^{-6}$ cm/s (geometric mean ± 95% CI), respectively, and 1.2 ± 0.5 (mean ± 95% CI) focal leaks per mm. Exposure to low concentrations (3 μM) of a cell-permeant analog of cAMP did not alter the barrier function. Exposure to higher concentrations (80 and 400 μM) and/or the phosphodiesterase inhibitor Ro-20-1724 (20 μM) lowered permeabilities and the number of focal leaks, and increased the selectivity of the barrier. Decreased permeabilities were accompanied by an increase in continuous VE-cadherin staining at cell–cell borders. Exposure to 1 mM 2′,5′-dideoxyadenosine, an inhibitor of adenylate cyclase, did not increase permeabilities. LECs expressed the lymphatic-specific master transcription factor Prox-1, regardless of whether barrier function was weak or strong. Our results indicate that the permeability of LEC tubes in vitro responds to cAMP in a manner similar to that well-described for the permeability of blood microvessels.

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Introduction

Regulation of macromolecular transport is a hallmark of proper microvascular function (Malik et al., 1989; Michel and Curry, 1999; Taylor and Granger, 1984). In vivo, the effective diffusional permeability for transport of bovine serum albumin (BSA) and similarly-sized dextrans across the endothelial wall is on the order of 10^{-8} cm/s in continuous capillaries and 10^{-8}–10^{-7} cm/s in venules (Garlick and Renkin, 1970; Gerlowski and Jain, 1986). This strong barrier results, in part, from tight junctions between blood microvascular endothelial cells (BECs) (Tsukita et al., 2001), the glycocalyx that surrounds these cells (Reitsma et al., 2007), basement membrane (Caulfield and Farquhar, 1974), factors secreted by mural cells and induced by heterotypic cell–cell contact (Thurston et al., 1999), and caveolae-limited transcytosis (Kohn et al., 1992; Milici et al., 1987). Less is known about the quantitative permeabilities of lymphatic microvessels, due to technical difficulties in cannulating initial lymphatic capillaries (Huber et al., 1984; Ono et al., 2005). Morphologically, junctions between lymphatic endothelial cells (LECs) take the form of interdigitated, overlapping, or abutting flaps that open to allow proteins to enter initial lymphatic microvessels (Baluk et al., 2007; Leak, 1971; Leak and Burke, 1966; Niiro and O'Morchoe, 1985; Schmid-Schönbein, 1990; Weber et al., 1991).

Recently, the isolation of LECs and BECs by specific surface markers (Kriehuber et al., 2001; Podgrabinska et al., 2002) has afforded the possibility of studying the regulation of barrier function of these two microvascular lineages in vitro. These cells maintain distinct patterns of gene expression, including the expression of many extracellular matrix proteins, cytokines, and growth factors (Hirakawa et al., 2003; Podgrabinska et al., 2002). Previous work on the barrier function of BECs in vitro – both with microvascular and large vessel-derived cells – has established the principle that the basal permeabilities of these cells are generally higher than observed in vivo (Albelda et al., 1988; Cooper et al., 1987; Wolburg et al., 1994). Despite this difference, in vitro cultures have proven quite useful for elucidating the mechanisms that regulate barrier function in BECs under well-defined conditions. The response of BECs to edemagenic agents generally shows similar trends in vitro and in vivo, although these effects may differ quantitatively (Garcia et al., 2001; Patterson et al., 1994). In particular, cyclic AMP (cAMP) appears to play a dominant role in regulating the barrier function of blood microvessels and BECs, and higher levels of intracellular cAMP correlate with increased tightness of the barrier (Adamson et al., 1998; Casnocha et al., 1989; He et al., 2000; Patterson et al., 1994; Schaeffer and Bitrick, 1995).

In this work, we measured the permeability of LEC tubes in vitro, as a first step towards understanding the regulation of lymphatic permeability by soluble agonists. We used a recently developed three-dimensional perfused model, in which ECs are cultured to confluence in a channel in a collagen gel (Chrobak et al., 2006). This particular geometry allows simultaneous measurement of effective diffusional permeabilities and localization of leaks. We found that the barrier function of LEC tubes responds to agents known to raise intracellular

concentrations of cAMP in a manner similar to that described for blood microvessels: Higher levels of cAMP tighten the lymphatic barrier, as indicated by decreased permeability coefficients, fewer numbers of focal leaks, and improved macromolecular selectivity.

Materials and methods

Cell culture

LECs from human dermal microvessels were obtained from Cambrex (lots 5F1013 and 5F1110). BECs from Cambrex (lot 5F1293) were used as a control. All cells were cultured as described previously (Chrobak et al., 2006).

Construction of microvascular tubes

Construction of microvascular tubes (128 in total) was essentially as previously described (Chrobak et al., 2006). Briefly, type I collagen (10 mg/mL from rat tail; BD Biosciences) was gelled around BSA-coated 120-μm-diameter stainless steel needles (Seirin). Removal of the needles yielded open cylindrical channels through collagen gels. Gels were conditioned for 24 h by perfusing them with culture media (MCDB 131 with 10% FBS, 1% glutamine–penicillin–streptomycin, 80 μM dibutyryl cAMP (db-cAMP), 1 μg/mL hydrocortisone, 2 U/mL heparin, 25 μg/mL ECGS, and 0.2 mM ascorbic acid 2-phosphate) that contained 3% 70 kDa dextran via wells at the inlet and outlet. LECs or BECs were trypsinized and seeded as a suspension into the channels, and grew to confluence by the next day. Tubes were fed continuously by perfusion at a flow of ~1 mL/h.

Measurement of permeabilities

On day 2 past seeding, tubes were refed with media containing 0, 3, or 80 μM db-cAMP, 0 μM db-cAMP plus 1 mM adenylate cyclase inhibitor 2′,5′-dideoxyadenosine (ddA; BioMol), or 400 μM db-cAMP plus 20 μM cAMP-specific phosphodiesterase inhibitor Ro-20-1724 (Calbiochem). All permeability measurements took place 24 h after refeeding, using previously described methods (Chrobak et al., 2006; Huxley et al., 1987): Alexa Fluor 594-conjugated BSA (50 μg/mL; Invitrogen) and Alexa Fluor 488-conjugated 10 kDa dextran (20 μg/mL; Invitrogen) were intro-duced through the lumen of an EC tube from an inlet reservoir. Fluorescence images were then taken after 20 min and 26 min in an environmental chamber held at 37 °C, using a Plan-Neo 10×/0.30 NA objective with flat-field correction (Axiovision 4.3; Zeiss). Effective diffusional permeability coefficients P_d were calculated from the equation $P_d = (1/\Delta I)(\delta I/\delta t)(d/4)$, where ΔI is the difference in averaged intensities of the 20-min and background images, δI is the difference between 26-min and 20-min images, δt is 6 min, and d is the diameter of the tube (Huxley et al., 1987). Control experiments verified that fluorescence signal was proportional to fluorophore concentration in the range of 0.2–50 μg/mL. Focal leaks ("leaky sites") were counted manually after maximizing image contrast, and are given as the number of leaks per frame per millimeter.

Immunofluorescence

Confluent LEC cultures were fixed in methanol for 10 min at 20 °C, blocked in 5% donkey serum (DS) for 60 min at 22 °C, and then incubated with rabbit anti-Prox-1 (1:1000; Upstate) and/or mouse anti-podoplanin (1:1000, clone D2-40; Signet) (Schacht et al., 2005) for 48 h at 4 °C. Cultures were re-washed with 5% DS, and then incubated with Alexa Fluor 594-conjugated donkey anti-rabbit and/or Alexa Fluor 488-conjugated donkey anti-mouse antibodies (1:500; Invitrogen) overnight at 4 °C. Nuclei were visualized with Hoechst 33342 (1 μg/mL) for 10 min at 22 °C. We counted the number of nuclei, Prox-1^{+} nuclei, and podoplanin^{+} cells in each 1.3 mm² field-of-view.

LEC tubes were fixed on day 3 or 4 past seeding. For immunostaining of PECAM and VE-cadherin, LEC tubes were fixed by perfusion with paraformaldehyde (4%, 20 min), and then permeabilized and blocked in 5% DS, 0.2% Triton X-100, and 10 mM glycine. For staining of Prox-1 and ZO-1, LEC tubes were fixed and permeabilized by perfusion with 20 °C methanol (20 min), and blocked with 5% DS. Tubes were perfused with primary antibody (2 h), washed (1 h), perfused with secondary antibody (1 h), and washed (3 h). Primary antibodies were: rabbit anti-Prox-1 (1:1000; Upstate), mouse anti-PECAM-1 (1:200, clone WM-59; Sigma), mouse anti-VE-cadherin (1:100, clone 75; BD Transduction Laboratories), and rabbit anti-ZO-1 (1:100; Zymed). Secondary antibodies were Alexa-conjugated donkey anti-IgGs (1:500). Confocal images were obtained with a PlanApo 10×/0.40 NA objective using an Olympus IX81 inverted microscope. Sequential images from the top to mid-plane of each tube were taken at 4.3 μm spacing, and stacked with ImageJ 1.38 (NIH).
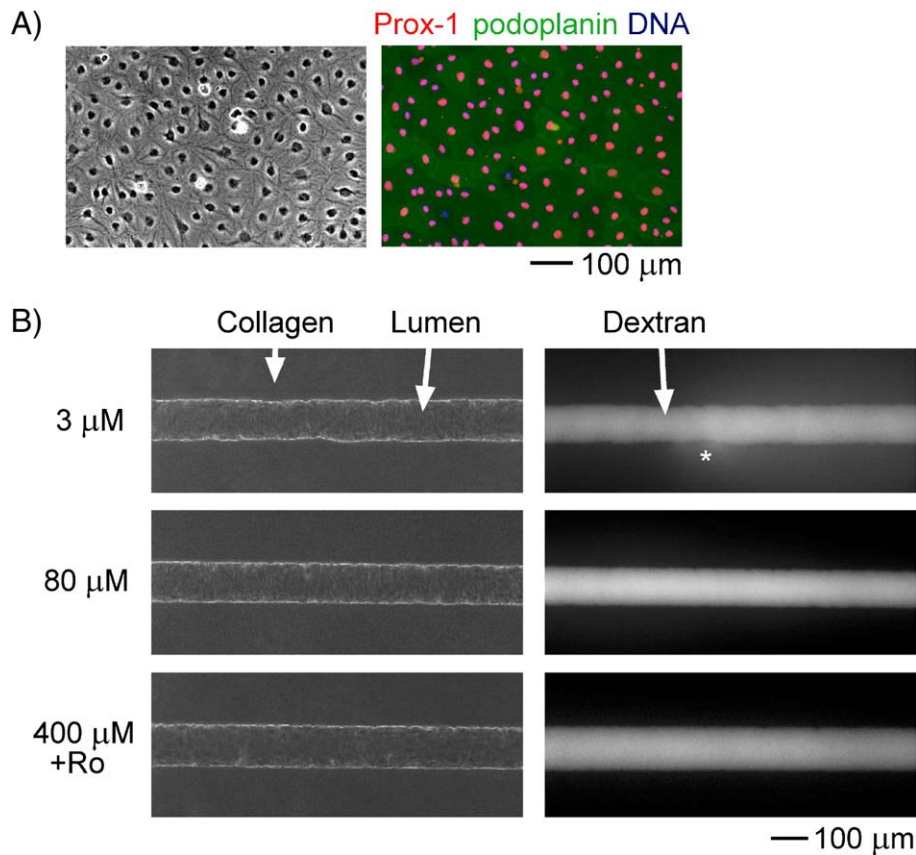

Fig. 1. LEC cultures and tubes. (A) Phase-contrast image of LECs, and fluorescence image of a culture stained for the lymphatic markers Prox-1 (red) and podoplanin (green) and DNA (blue). (B) Phase-contrast image of LEC tubes cultured under 3 μM db-cAMP, 80 μM db-cAMP, and 400 μM db-cAMP + 20 μM Ro-20-1724 (Ro), and fluorescence images after 26 min of perfusion with Alexa 488-conjugated 10 kDa dextran. A focal leak (indicated by asterisk) is present in the 3 μM db-cAMP tube. LEC tubes cultured under 0 μM db-cAMP appeared similar to those under 3 μM db-cAMP.

Quantifying VE-cadherin junctions

Junctional staining for VE-cadherin was quantified by measuring the average lengths of uninterrupted VE-cadherin staining in tubes perfused with 3 μM db-cAMP, 80 μM db-cAMP, or 400 μM db-cAMP plus Ro-20-1724. Data were averaged over 3 tubes per condition (≥35 cells per tube).

Statistical analysis

Comparisons used the Mann–Whitney *U* test (Prism 5; GraphPad), unless noted otherwise; differences were considered significant when $P<0.01$. Comparisons of VE-cadherin staining used the unpaired *t*-test, with differences considered significant when $P<0.05$.

Permeability coefficients are presented as geometric means±95% CI, and all other data as arithmetic means±95% CI.

Results

Characterization of LEC cultures

To verify the purities of the cell cultures, we stained them for Prox-1 and podoplanin, which are selectively expressed in LECs in vitro and in vivo (Hirakawa et al., 2003; Petrova et al., 2002; Podgrabinska et al., 2002; Wigle et al., 2002). The two lots of LECs were >97% positive for Prox-1 and podoplanin (Fig. 1A). Positive signals for Prox-1 always overlapped with those for podoplanin. Cell–cell junctions were clearly defined in LECs (Fig. 1A). BECs were >93% negative for Prox-1 and podoplanin.

Permeabilities of LEC tubes

When seeded into collagen channels, LECs rapidly attached, spread, and proliferated to form confluent monolayers after 1 day. By phase-contrast microscopy, endothelial morphology and junctions were less distinct in tubes than on plastic dishes (Fig. 1B).

Using time-lapse fluorescence microscopy at day 3 post-seeding, we found that LEC tubes exhibited a weak barrier to the passage of BSA or dextran across the wall (Figs. 1 and 2) in the absence of db-cAMP. The diffusional permeability coefficients to BSA (P_{BSA}) and 10 kDa dextran (P_{dex}) were $1.4^{+0.9}_{-0.6}\times 10^{-6}$ cm/s and $1.7^{+0.8}_{-0.5}\times 10^{-6}$ cm/s, respectively. The number of focal leaks was high (1.2±0.5/mm), and the selectivity of the barrier – defined as P_{dex}/P_{BSA} – was correspondingly poor (1.2±0.2). A small amount of db-cAMP (3 μM) did not alter the barrier: P_{BSA} and P_{dex} remained high at $1.2^{+0.5}_{-0.4}\times 10^{-6}$ cm/s ($P=0.7$, compared to tubes cultured without db-cAMP) and $1.4^{+0.6}_{-0.4}\times 10^{-6}$ cm/s ($P=0.6$), respectively. Similarly, the number of focal leaks (1.0±0.3/mm, $P=0.4$) and the selectivity (1.2±0.2, $P=0.9$) did not change.

In blood microvessels, agents that increase intracellular concentrations of cAMP reduce the number of leaks and decrease macromolecular permeability coefficients (Adamson et al., 1998; He et al., 2000). To see whether lymphatic microvessels responded similarly, we measured the barrier function of LEC tubes that were treated with higher concentrations of db-cAMP (which readily passes the cell membrane) and/or the phosphodiesterase inhibitor Ro-20-1724, which is known to further increase levels of intracellular cAMP by inhibiting degradation (Rubin et al., 1991). In tubes supplemented with 80 μM db-cAMP, P_{BSA} decreased to $3.0^{+1.0}_{-0.7}\times 10^{-7}$ cm/s ($P<0.0001$, compared to tubes treated with 3 μM db-cAMP), P_{dex} decreased to $6.2^{+0.9}_{-0.8}\times 10^{-7}$ cm/s ($P<0.0001$), and the number of focal leaks decreased greatly (0.05±0.06/mm, $P<0.0001$). Selectivity of the barrier increased to 2.2±0.5 ($P<0.0001$).

Addition of 400 μM db-cAMP and 20 μM Ro-20-1724 resulted in further enhancement of barrier function: P_{BSA} decreased to $1.1^{+0.3}_{-0.3}\times 10^{-7}$ cm/s ($P<0.0001$, compared to tubes treated with 80 μM db-cAMP), P_{dex} decreased to $3.8^{+0.3}_{-0.3}\times 10^{-7}$ cm/s ($P<0.0001$), and selectivity increased to 3.8±1.0 ($P=0.0008$). We did not observe focal leaks under these conditions.

We also measured the permeability of LEC tubes perfused with 1 mM ddA and 0 μM db-cAMP. Addition of ddA, an inhibitor of adenylate
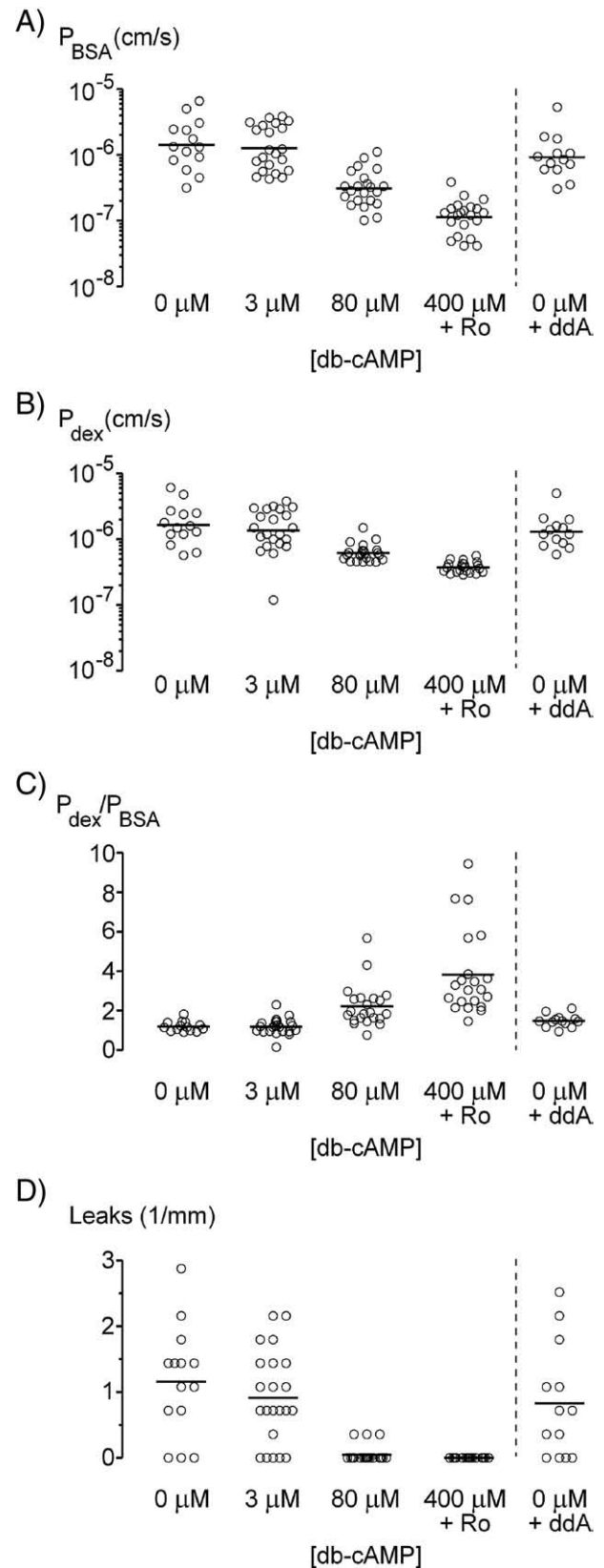

Fig. 2. Permeabilities of LEC tubes to BSA and 10 kDa dextran at day 3 post-seeding. (A) BSA permeability coefficients for LEC tubes treated with 0 μM db-cAMP ($n=14$), 3 μM db-cAMP ($n=22$), 80 μM db-cAMP ($n=22$), 400 μM db-cAMP+20 μM Ro-20-1724 ($n=21$), and 0 μM db-cAMP+1 mM ddA ($n=13$). (B) 10 kDa dextran permeability coefficients for LEC tubes. (C) Size selectivity of LEC tubes, defined as the ratio of permeability to 10 kDa dextran over the permeability to BSA. (D) The occurrence of focal leaks per millimeter in LEC tubes.
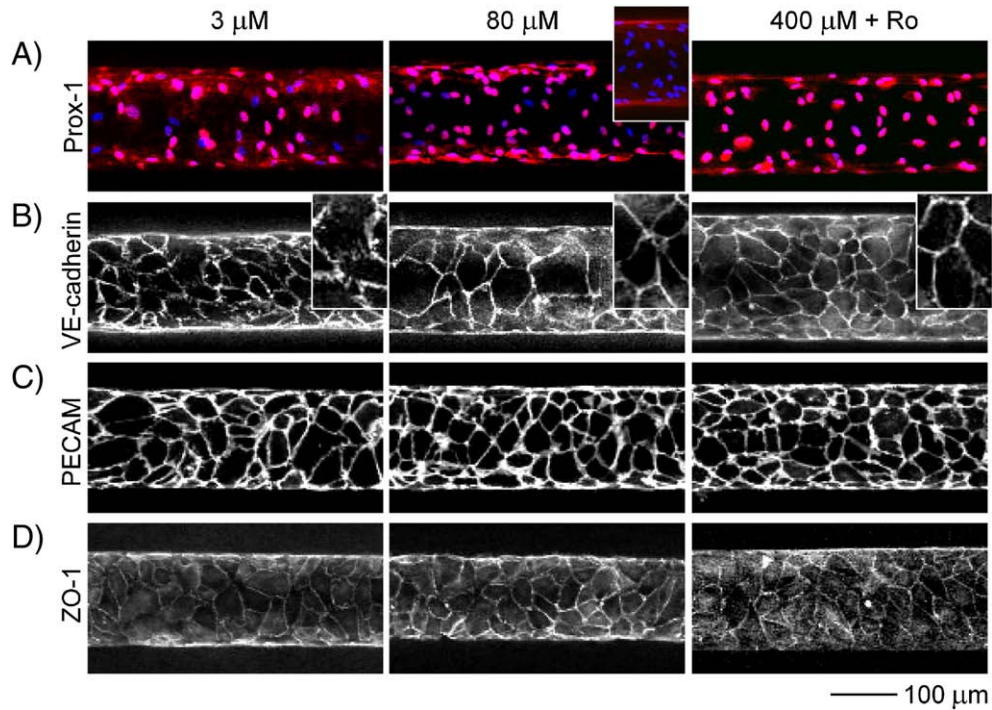
Fig. 3. Expression of Prox-1 and junctional markers in LEC tubes. (A) Prox-1 (false color red) and DNA (blue). *Inset*, negative Prox-1 staining in a BEC tube. (B) VE-cadherin. *Insets*, VE-cadherin staining magnified 1.8×. (C) PECAM. (D) ZO-1. Images are representative of three separate experiments.

cyclase, has been shown to weaken the hydraulic barrier of explanted venules, most likely by decreasing intracellular levels of cAMP (He et al., 2000; Holgate et al., 1980). In LEC tubes, however, perfusion with ddA had little effect: P_{BSA} and P_{dex} were $0.9^{+0.5}_{-0.3} \times 10^{-6}$ cm/s ($P=0.2$, compared to tubes treated with 0 μM db-cAMP) and $1.3^{+0.5}_{-0.4} \times 10^{-6}$ cm/s ($P=0.3$), respectively. The occurrence of focal leaks did not change (0.8 ± 0.5/mm, $P=0.3$). Surprisingly, the selectivity of the barrier increased slightly over baseline conditions (1.5 ± 0.2, $P=0.012$).

Expression of lymphatic and junctional markers in LEC tubes

To determine whether the observed effects resulted from over-growth of LEC tubes with a small population of BECs, we stained tubes *in situ* for Prox-1. We found that Prox-1 was expressed in nuclei regardless of the amount of db-cAMP (Fig. 3A). Tubes grown under 3 μM db-cAMP displayed short and non-uniform junctional staining for VE-cadherin, a marker for adherens junctions (Lampugnani et al., 1995); on average, segments of continuous VE-cadherin staining measured 19.0 ± 5.8 μm in length. In contrast, tubes grown under 80 μM db-cAMP or 400 μM db-cAMP plus Ro-20-1724 showed clearly defined junctions (Fig. 3B and insets) that had longer segments of continuous staining: 29.4 ± 5.7 μm for 80 μM db-cAMP ($P=0.0054$, compared with tubes treated with 3 μM db-cAMP), and 29.0 ± 10.3 μm for 400 μM db-cAMP plus Ro-20-1724 ($P=0.022$).

We did not observe any qualitative or quantitative differences in staining for PECAM-1 (Fig. 3C) and ZO-1 (Fig. 3D), a marker of tight junctions (Stevenson et al., 1986). In all cases, PECAM-1 localized strongly at junctions, while ZO-1 exhibited junctional as well as diffuse expression. Control staining demonstrated absence of Prox-1 in BEC tubes (Fig. 3A, inset).

Discussion

Our results indicate that the barrier function of human LEC tubes in vitro responds to cAMP, a second messenger known to affect the permeability of blood microvessels in part via control of actomyosin

dynamics (Adamson et al., 1998; Dudek and Garcia, 2001). This work provides a first step towards understanding how the permeability of lymphatic microvessels is regulated. In particular, exposure to agents that increase intracellular concentrations of cAMP enhanced barrier function in LEC tubes to a remarkable degree. In the presence of large amounts of dibutyryl cAMP and the cAMP-specific phosphodiesterase inhibitor Ro-20-1724, permeability coefficients to BSA in several LEC tubes reached low values comparable to those reported for blood microvessels (i.e., on the order of $5-10 \times 10^{-8}$ cm/s). Under these conditions, we observed little functional difference between BEC and LEC tubes in vitro. LEC tubes continued to express the lymphatic marker Prox-1, suggesting that de-differentiation or transdifferentiation of LECs to a BEC phenotype (Amatschek et al., 2007) was incomplete.

Addition of the adenylate cyclase inhibitor ddA did not worsen the barrier of LEC tubes. In blood microvessels, ddA weakens barrier function, presumably because basal generation of cAMP by adenylate cyclase is substantial in BECs (He et al., 2000). Our data suggest that the basal activity of adenylate cyclase in LEC tubes is insufficient to affect lymphatic permeability.

A major advantage of measuring permeability in a tubular, rather than planar, geometry is the ability to view the vessel wall in profile. Using this perspective, we determined that the amount of extravasated BSA depended on both losses at focal leaks and uniform leakage along the lengths of the tubes. The effect of increased concentrations of cAMP appears to be two-fold in our system. First, moderate amounts of db-cAMP (80 μM) led to the nearly complete disappearance of focal leaks. Second, large amounts (400 μM, with Ro-20-1724) led to improved selectivity of the barrier and decrease in the uniform leakage along tubes.

We believe that changes in permeability primarily reflect the presence or absence of junctional gaps, as shown by immunofluorescence staining. With 3 μM db-cAMP, we observed discontinuous expression of VE-cadherin at cell–cell junctions, while higher concentrations yielded stronger or more continuous expression of VE-cadherin at borders. Our observations are consistent with previous studies showing that cAMP enhances VE-cadherin-mediated intercellular adhesion in BECs (Dye et al., 2001; Koch et al., 2000; Waschke et al., 2004). LEC tubes strongly

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expressed PECAM-1 and weakly expressed ZO-1, a marker of tight junctions in endothelia (Lampugnani and Dejana, 1997; Stevenson et al., 1986), and we could not detect any quantitative difference in staining intensities under different concentrations of db-cAMP. Surprisingly, ZO-1 is present at junctions in lymphatic microvessels in vivo (Baluk et al., 2007); whether cAMP plays an important role in regulating its expression and localization as it does in BECs (Adamson et al., 1998; Dye et al., 2001; Koch et al., 2000; Rubin et al., 1991; Wolburg et al., 1994) is unclear. Definitive assessment of LEC junctions and other structures relevant for permeability (caveolae and glycocalyx) will require imaging with electron microscopy.

We are unaware of any studies that have examined the effect of cAMP on lymphatic permeability in vivo. The concentration of cAMP in circulating plasma is on the order of 10–50 nM (Rabinowitz and Katz, 1973), and one would not expect lymph to have high concentrations of cAMP or its analogues. This condition and the observed leakiness of lymphatic microvessels in vivo (Zweifach and Prather, 1975) are consistent with our finding that low concentrations of db-cAMP correlates with a leaky LEC barrier. Interventions that increase circulating concentrations of cAMP, such as the vascular introduction of rolipram or forskolin for treatment of ischemia–reperfusion injury, are believed to attenuate edema through improved barrier function of blood microvessels (Adkins et al., 1992; Barnard et al., 1994; Seibert et al., 1992). If the trends implied by our in vitro study are present in vivo, then these therapies may have an unintended secondary effect of enhancing the barrier of surrounding lymphatics, with the potential to impede lymphatic drainage.

Overall, our results support the idea that cAMP improves the barrier function of lymphatic microvessels in vitro. In particular, LEC tubes – which exhibited many leaks under basal and low cAMP conditions – responded to agents that increase concentrations of cAMP with decreases in permeability coefficients and the number of focal leaks and an increase in selectivity. These changes occurred amidst the enhanced expression of VE-cadherin and the continued expression of the lymphatic marker Prox-1 and the junctional markers PECAM-1 and ZO-1. Further work in this area may potentially shed light on how blood and lymphatic microvessels can exhibit such distinct barrier phenotypes in vivo, the role that cAMP and other messengers play in this regulation, and how to engineer functionally leaky lymphatics in vitro.

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