Growth factor- and heparin-dependent regulation of constitutive and agonist-mediated human endothelial barrier function

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1Cellular Engineering Technologies, Inc., 2Departments of Biomedical Engineering and 3Pathology, University of Iowa, Iowa City, Iowa; 5Department of Medical Physiology, Texas A & M University Health Science Center, Temple, Texas; and 4Department of Surgery, University of California Davis Medical Center, Sacramento, California

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Moy, Alan B., Ken Blackwell, Mack H. Wu, and Harris J. Granger. Growth factor- and heparin-dependent regulation of constitutive and agonist-mediated human endothelial barrier function. Am J Physiol Heart Circ Physiol 291: H2126–H2135, 2006. First published May 5, 2006; doi:10.1152/ajpheart.00185.2006.—We report functional differences in constitutive and agonist-mediated endothelial barrier function between cultured primary and Clonetics human umbilical vein endothelial cells (pHUVEC and cHUVEC) grown in soluble growth factors and heparin. Basal transendothelial resistance (TER) was much lower in pHUVEC than in cHUVEC grown in medium supplemented with growth factors, such as basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and human epithelial growth factor (EGF), and heparin. On the basis of a numerical model of TER, the increased basal TER in cHUVEC was due to effects on cell-matrix adhesion and membrane capacitance. Heparin and bFGF increased constitutive TER in cultured pHUVEC, and heparin mediated additional increases in constitutive TER in pHUVEC supplemented with bFGF. EGF attenuated bFGF-mediated increases in TER. On the basis of the numerical model, in contrast to cHUVEC, heparin and bFGF augmented TER through effects on cell-cell adhesion and membrane capacitance in pHUVEC. Thrombin mediated quantitatively greater amplitude and a more sustained decline in TER in cultured cHUVEC than in pHUVEC. Thrombin-mediated barrier dysfunction was attenuated in pHUVEC conditioned in EGF in the presence of bFGF. cAMP stimulation mediated differential attenuation of thrombin-mediated barrier dysfunction between pHUVEC and cHUVEC. VEGF displayed differential effects in TER in serum-free medium. Taken together, these data demonstrate marked differential regulation of constitutive and agonist-mediated endothelial barrier function in response to mitogens and heparin stimulation.

commercial endothelial; fibroblast growth factor; vascular endothelial growth factor; epithelial growth factor; numerical modeling of transendothelial resistance

DEMAND FOR AND UTILIZATION OF commercial human endothelial cells for high-throughput cell-based drug screening in preclinical drug discovery have increased (6, 15, 25, 26). To meet this demand, there has been an increased urgency to greatly expand the supply and yield of human cultured endothelial cells that are isolated from vascular tissue. Cultured human endothelial cells typically have greater and more complex growth requirements than endothelial cells derived from animal tissues. To meet this increased demand, cultured human endothelial cells are grown in a number of growth factors or mitogens and supplements to increase and sustain cell proliferation. Although commercial human endothelial cell lines are certified free of pathogens and characterized as endothelium entirely on the basis of their morphological characteristics, the presence of acetylated LDL and factor VIII biomarkers, and the absence of smooth muscle contamination, physiological parameters of endothelial function are not routinely evaluated and are not part of the certification standards. More importantly, the mechanism by which mitogens modulate endothelial function has not been systematically evaluated.

Little attention has been given to whether the functional properties of commercial endothelial cell lines preserve or approximate the normal physiological responses of institutional primary cell isolates. If culture condition represents the dominant variable that defines endothelial function, then predictions of drug responses from commercial secondary human endothelial cells will not extrapolate responses of primary human cell isolates and could prove misleading in predicting in vivo responses.

More problematic is the use of commercial human endothelial cells to elucidate generalized mechanisms of molecular signal transduction in vascular biology (2, 12, 31). There is increased interest in utilizing human endothelial cells to predict human in vivo vascular biology. However, if growth factors and experimental culture conditions dominate normal physiological responses in commercial cell lines, then such experimental data may misrepresent normal physiological responses of vascular biology. Also, if there is a lack of awareness of intrinsic endothelial properties and function, resolution of conflicting data on molecular signal transduction without physiological standardization of in vitro endothelial biology is problematic.

The differential and unique impact of growth factors and mitogens on endothelial barrier function has not been well documented by controlled studies. However, there is documented evidence that some growth factors remodel the endothelial cytoskeleton, which could impact endothelial function. For example, fibroblast growth factor (FGF) mediates differential expression of integrin receptors on cultured microvascular endothelial cells (14). Plopper et al. (27) reported that FGF-dependent signal transduction converges at focal adhesion complexes in cultured microvascular endothelial cells. Vascular endothelial growth factor (VEGF) induces microvascular permeability in the setting of angiogenesis or in the pathogenesis of tumor metastasis (7, 18, 32). VEGF activates...
Src activity, which leads to a disruption of VE-cadherin and β-catenin binding (32). Wu et al. (33) reported activation of focal adhesion kinase in VEGF-stimulated cultured human umbilical vein endothelial cells (HUVEC), suggesting a potential mechanical coupling between VEGF and remodeling of cell-matrix adhesion. These data implicate growth factors as a potential determinant that regulates endothelial barrier function.

Heparin induces endothelial proliferation in FGF-treated cultured endothelial cells (13) and augments barrier function in cultured human endothelial cells exposed to bovine brain extract (1). A number of reports have documented limitation of cancer metastasis in several animal cancer models by heparin or heparin-like molecules (4, 8, 17, 28, 29, 34). Ludwig et al. (17) recently reported a heparin-associated reduction in melanoma metastasis accompanied by endothelial P-selectin expression. However, it is not well documented whether heparin has a direct impact on endothelial membrane properties, and it is not known whether heparin interacts cooperatively or antagonistically on endothelial barrier function with other mitogens or edemagenic stimuli. Because heparin is used to clinically treat cardiovascular disorders, a precise understanding of the impact of heparin on constitutive and agonist-mediated endothelial barrier function is critical.

We previously reported several characteristic static, dynamic, and quantitative physiological parameters of endothelial function in cultured primary HUVEC (pHUVEC) grown in the absence of growth factors (19–21, 23, 24, 30). One measurement, in particular, quantifies endothelial barrier function on the basis of the measurement of transendothelial resistance (TER) across a cultured monolayer grown on a microelectrode (23). This technique can dynamically quantify endothelial barrier function and cell motility at the same resolution as transmission electron microscopy, but in a more efficient and cost-effective manner, in living cultured endothelial monolayers (24). The technique captures the nonlinear behavior of cultured endothelial cells in response to physiological stimuli. We previously reported that thrombin transiently decreases TER in cultured HUVEC (23). An actin-myosin-independent process regulates the initial and rapid decline in TER, whereas the restoration of TER is dependent, in part, on myosin light chain-dependent force generation. We also reported that agents that increase intracellular cAMP do not prevent the decline in TER but rapidly accelerate the restoration of thrombin-mediated barrier dysfunction independent of inhibition of myosin light chain-dependent force generation (20). We also used the measured TER to develop computer-based numerical models of cytoskeletal membrane properties in cultured cells (10, 19, 22, 24). The computer algorithms resolve measurements of TER into measurements of cell-cell adhesion (R₀), cell-matrix adhesion (α), and membrane capacitance (C_m). Additionally, these computer algorithms calculate the error of the numerical analysis, which provides an assessment of model stability and reliability (3).

To our knowledge, there have been few controlled studies that precisely document the impact of soluble mitogens on constitutive and agonist-mediated endothelial barrier dysfunction. Here, we report marked quantitative and qualitative functional differences in constitutive and agonist-mediated parameters of TER between cultured pHUVEC grown in the presence and absence of growth factors and heparin and commercial (Clonetics) HUVEC (cHUVEC), which are typically grown in the presence of several growth factors. Using a numerical model that resolves transcellular impedance into R₀ and α, we report marked differences in cytoskeletal membrane properties between cultured pHUVEC and cHUVEC (19, 22, 24). We document complete alterations in dynamic responses in TER between cultured cHUVEC and pHUVEC in response to thrombin, cAMP analogs, and the angiogenic stimulus VEGF. Furthermore, we document the impact of mitogens on constitutive and agonist-mediated endothelial barrier function in cultured pHUVEC. This report demonstrates that the standard markers and morphological characteristics routinely used to define cultured endothelial cells are not sufficient to predict functional properties of cultured endothelial cells under static and dynamic physiological conditions. The outcome of these physiological responses in endothelial function has significant implications and poses challenges for extrapolation of molecular mechanisms of signal transduction from commercial endothelial cell lines to primary endothelial cell isolates. This report provides further insight into treatment strategies that utilize mitogens and heparin to modulate endothelial barrier function for vascular disorders.

MATERIALS AND METHODS

Materials. Human α-thrombin was obtained from Enzyme Research Laboratories (South Bend, IN). Human VEGF (VEGF-165, catalog no. 293-VE-010) was purchased from R & D Systems (Minneapolis, MN) for experiments depicted in Fig. 6 and from Sigma Chemical (St. Louis, MO) for all other experiments. Microelectrodes were obtained from Applied Biophysics (Troy, NY). Human EGF, basic FGF (bFGF), theophylline, and forskolin were purchased from Sigma Chemical and heparin sodium from American Pharmaceutical Partners (Schaumburg, IL).

Cell culture. Primary isolated cultured HUVEC were prepared by collagenase treatment of freshly obtained human umbilical veins by the University of Iowa Tissue Culture Facility, as described previously (5). Harvested primary cultures designated for cell adhesion assays were plated on 60-mm tissue culture plates that were coated with fibronectin (100 μg/ml). Cultures were identified as endothelial by their characteristic uniform morphology, uptake of acetylated LDL, and indirect immunofluorescent staining for factor VIII.

cHUVEC (single donor) were obtained from Clonetics, which is currently known as Cambrex (Walkersville, MD). The cells were grown in EGM-2 containing human epithelial growth factor (EGF), hydrocortisone, 6% fetal bovine serum, VEGF, human FGF-B (with heparin), ascorbic acid, insulin growth factor, and GA-1000 (gantamicin and amphotericin B). Cambrex protects the concentration of all growth factors under trade secrets. Constitutive and agonist-mediated endothelial barrier function studies were conducted after a confluent density was inoculated on a microelectrode and monitored for 24 h.

In other studies, TER was measured in pHUVEC over a 60-h period in the presence of an individual growth factor or a combination of growth factors or heparin in M199 containing 20% serum. The medium was then removed and replaced with M199 containing BSA (10 mg/ml), and the cultured monolayers were exposed to thrombin for evaluation of the physiological response of barrier function to prolonged conditioning in a different medium.

Measurement of endothelial barrier function by TER. Endothelial barrier function was measured using the electrical cell substrate...
sensing (ECIS) technique (Applied Biophysics) (23). The cells were cultured on a small (5 × 10⁻⁴ cm²) gold electrode, with culture medium used as the electrolyte, and barrier function was measured dynamically at 4,000 Hz by determination of the electrical impedance of a cell-covered electrode. With the use of proprietary algorithms (Applied Biophysics), the ECIS system reports the resistance and capacitance from the measured in-phase and out-phase voltage with the assumption that the circuit of a cell-covered electrode is a resistor and capacitor in series. Barrier integrity was expressed as fractional change in TER in experiments in which cultured monolayers were challenged with physiological stimuli. In some cases, barrier function was expressed as total resistance or real value of impedance of the cell monolayer, as previously described by Haxhinasto et al. (10).

Breakdown of cytoskeletal membrane properties by numerical modeling of experimental transcellular impedance. A numerical analysis was used to calculate specific cell-cell and cell-substrate adhesion and \( C_m \) on the basis of the measured transcellular impedance recorded by the ECIS system using a model described elsewhere (19, 22, 24) and determined by computer algorithms recently described in complete detail elsewhere (3). Briefly, the total impedance across a cell-covered electrode is composed of the impedance created between the ventral surface of the cell and the electrode (\( \alpha \)), the impedance created between cells (\( R_b \)), the transcellular impedance created from transcellular current conduction (\( Z_m \)), and the impedance of a naked electrode (\( Z_a \)). For these calculations, \( Z_m \) is inversely related to \( C_m \), which is dependent on membrane convolution, which, in turn, is dependent on the cortical cytoskeleton. The real and imaginary experimental data of the cell-covered and naked electrodes were measured at frequencies of 25–60,000 Hz. The impedance of the cell-covered electrode (\( Z_c \)) used in the model was measured 24 h after cell attachment, when the endothelium achieved a steady-state TER. \( Z_m \) was measured after treatment of cultured monolayers with trypsin and replacement of the medium with fresh medium. A calculated real and imaginary value (\( Z \)) was generated from the solutions of \( \alpha \), \( R_b \), and \( C_m \), obtained from a multiresponse Levenberg-Marquardt nonlinear optimization model of the real data only. The difference between the calculated and experimental cell impedance was defined by the following expression: \( Z_{error} = (Z_{measured}/Z_{errors}) - 1 \), which was expressed as a function of current frequency. An error evaluation was calculated using a \( \chi^2 \) analysis of the least squared sum of the calculated and experimental residuals as a function of current frequency and was normalized to the number of data points. A frequency bandwidth was selected to find the optimal solutions of \( \alpha \), \( R_b \), and \( C_m \) with the lowest \( Z_{error} \) and with the lowest normalized \( \chi^2 \) for each experiment.

Statistical analysis. Values are means ± SE. Comparisons between groups were made using an unpaired Student’s t-test. Comparisons between more than two groups were made using an analysis of variance. Individual group comparisons were done using Tukey’s honest significant difference test for post hoc comparison of means. Differences were considered significant at \( P \leq 0.05 \).

RESULTS

Basal TER is greater in cultured cHUVEC than pHUVEC. A confluent monolayer of cultured endothelial cells was inoculated on a microelectrode, a minute alternating current was applied across the circuit, and TER and capacitance were continuously monitored. During the first 15 h of attachment on the microelectrode (Fig. 1), the basal steady-state TER was ~50% higher in unstimulated cultured cHUVEC than pHUVEC.

As shown in the frequency-resistance spectrum in Fig. 2, \( Z_c \) was higher than \( Z_a \) at all frequencies except <1,000 Hz in cultured pHUVEC, which indicates a systematic alteration in the conductive properties at low frequencies in the naked electrode after trypsin exposure. In contrast, \( Z_c \) was higher than \( Z_a \) at all frequencies in cultured cHUVEC. When normalized to \( Z_m \), the data indicate the frequency at which there was a maximal measured \( Z_c \) and \( Z_a \) in cultured pHUVEC (Fig. 2C) and cHUVEC (Fig. 2D). Again, the data show that \( Z_c \) is lower than \( Z_a \) at frequencies <1,000 Hz in cultured pHUVEC but that \( Z_c \) is greater than \( Z_a \) at all frequencies in cultured cHUVEC.

There were marked differences in how the numerical model predicted the measured \( Z_c \), which was expressed by the calculated \( Z_{error} \) in cultured pHUVEC (Fig. 2E) and cHUVEC (Fig. 2F). The calculated \( Z_{error} \) for cultured pHUVEC was greatest at <1,000 Hz (20% error), and the value was negative, because \( Z_c \) was lower than \( Z_a \) after trypsinization. However, \( Z_{error} \) was only 1% at >1,000 Hz in cultured pHUVEC, indicating that the numerical model provided an excellent prediction of the experimental TER. In contrast to cultured pHUVEC, cultured cHUVEC produced a positive (~20%) \( Z_{error} \) at <1,000 Hz, because \( Z_c \) was higher than \( Z_a \) measured after exposure to trypsin. Also, in contrast to cultured pHUVEC, cultured cHUVEC exhibited a positive (~20%) \( Z_{error} \) at high current frequency. Compared with cultured pHUVEC, these data indicate that the model identified the best solutions of the experimental data only when a narrower bandwidth of the experimental data was modeled in cultured cHUVEC.

Using this numerical model, which breaks down the measured TER into \( R_b \), \( \alpha \), and \( C_m \), we observed significant differences in cytoskeletal membrane properties between cultured pHUVEC and cHUVEC (Fig. 3). \( R_b \) was not significantly different between the two cell lines. However, \( \alpha \) was significantly greater in cultured cHUVEC, indicating greater \( \alpha \) in cultured cHUVEC than pHUVEC. Additionally, the computational algorithms identified significant decreases in \( C_m \), which would result in a greater \( Z_m \). Taken together, these data document that the difference in TER between cultured cHUVEC and pHUVEC was attributed to differences in \( \alpha \) and \( C_m \).
Another way to characterize changes in endothelial phenotype is to evaluate whether there is a shift in the frequency spectrum between cultured pHUVEC and cHUVEC. We observed a rightward shift in the peak frequency of $Z_c$ (Fig. 2, C and D) in cultured cHUVEC compared with pHUVEC. Peak frequency was $6,250 \pm 289$ and $7,000 \pm 471$ Hz for cultured pHUVEC and cHUVEC, respectively. However, this difference did not achieve statistical significance ($P = 0.1$).

Physiological stimuli induced different TER responses in cultured pHUVEC and cHUVEC. Thrombin induced marked quantitative and dynamic differences in TER in cultured cHUVEC and pHUVEC (Fig. 4). Thrombin mediated a rapid but transient decline in TER in cultured pHUVEC, which illustrates the nonlinear effect of physiological stimuli on endothelial barrier function. However, the same dose of thrombin mediated a greater and more sustained decline in cultured cHUVEC than pHUVEC.

We previously reported that theophylline- and forskolin-induced increases in intracellular cAMP did not prevent the thrombin-mediated decline in TER in cultured pHUVEC, but increases intracellular cAMP did accelerate the restoration of TER to initial basal levels (20). In contrast, theophylline and forskolin pretreatment completely inhibited the thrombin-mediated decline in TER in cultured cHUVEC. Instead, thrombin mediated a modest enhancement in barrier function (Fig. 5). Taken together, the signal transduction mechanisms by which cAMP agonists protect against thrombin-mediated barrier dysfunction are clearly different between cultured pHUVEC and cHUVEC.

The effects of VEGF on TER were markedly different between cultured pHUVEC and cHUVEC (Fig. 6). VEGF...
mediated a slow decline in TER in cultured pHUVEC, whereas it caused a very different biphasic response in cultured cHUVEC. In cultured cHUVEC, VEGF caused a rapid but transient decline in TER and mediated a subsequent increase in TER to above initial basal levels. This response is similar to our previously reported response to histamine in cultured pHUVEC (23). The marked differential response of barrier function to VEGF reinforces the notion that the response of endothelial function to physiological stimuli is impacted by culture conditions that remodel endothelial phenotype.

**Impact of heparin on regulation of constitutive and thrombin-mediated endothelial barrier function.** A confluent density of cultured pHUVEC was inoculated in the absence or presence of heparin in medium containing serum, and TER was measured as a function of a postconfluency state (Fig. 7). Heparin at 0.25–10 U/ml mediated a near dose-dependent increase in TER over a 60-h period compared with control pHUVEC monolayers; heparin at 50 U/ml mediated a slight submaximal increase in TER over the same time period.

Thrombin-mediated barrier dysfunction was attenuated in cultured pHUVEC monolayers exposed to heparin at 0.25 U/ml for 60 h (Fig. 8). This concentration was chosen because it approximated the calculated blood concentration for a therapeutic dose of heparin based on an average blood volume of 5 liters, with a therapeutic dose of 1,200 U/h. In these and subsequent thrombin-stimulated experiments, the conditioned medium was replaced with serum and heparin-free medium.

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**Fig. 3.** Static differences in measured cell membrane properties between pHUVEC and cHUVEC. Numerical model of TER was used to measure cell-cell adhesion ($R_b$), cell-matrix adhesion ($\alpha$), and membrane capacitance ($C_m$). Values are means ± SE for a sample size of 4 experiments in absolute values: $\Omega^0.5$·cm for $\alpha$, $\Omega$·cm$^2$ for $R_b$, and $\mu$F/cm$^2$ for $C_m$. *Significantly different from pHUVEC ($P < 0.05$).
Thrombin-mediated endothelial barrier function.

Heparin enhanced constitutive endothelial barrier function together, these data document that therapeutic concentrations of heparin for 60 h, the effect of thrombin on endothelial barrier function was not modified. Taken together, these data document that heparin mediates thrombin-mediated barrier dysfunction.

Impact of growth factors and heparin on constitutive and thrombin-mediated endothelial barrier function. TER was monitored over a period of several hours in postconfluent cultured pHUVEC exposed to medium containing growth factors in the presence or absence of heparin (Fig. 9). A confluent density of cultured pHUVEC was inoculated on microelectrodes in medium containing serum and a defined amount of growth factors, and TER was subsequently monitored over time. TER in VEGF-stimulated cultured pHUVEC at 24, 48, and 60 h was not statistically different from that in control monolayers. In monolayers exposed to EGF, no statistical difference in TER was observed at 24, 48, and 60 h compared with control monolayers. In contrast, TER in monolayers exposed to bFGF was substantially increased at 24, 48, and 60 h compared with that in control monolayers.

Additionally, the antagonistic effects on constitutive TER were different among growth factors in confluent pHUVEC (Fig. 9). EGF attenuated bFGF-mediated increases in TER in cultured pHUVEC at 24 and 48 h compared with monolayers exposed to FGF only. Addition of VEGF had no additional impact on TER in cultured pHUVEC monolayers exposed to EGF and bFGF.

TER was modified by addition of heparin to monolayers in the presence of some growth factors (Fig. 9). Heparin in the presence of EGF decreased constitutive barrier function of cultured monolayers only at 24 h compared with monolayers exposed to EGF only. Additionally, heparin mediated additional increases in constitutive TER at 24 and 60 h in monolayers exposed to bFGF compared with monolayers exposed to bFGF only. However, TER was significantly lower in cells exposed to heparin and bFGF than in cells exposed only to bFGF at 24 h.

In bFGF-stimulated monolayers, heparin at 0.25–10 U/ml augmented constitutive TER in a dose-dependent fashion over a 60-h period (Fig. 10). Compared with monolayers exposed to bFGF only, heparin further increased TER. Also, TER was significantly higher in monolayers exposed to 10 U/ml heparin in the presence of bFGF than in monolayers exposed to bFGF and 0.25 U/ml heparin.

Cultured monolayers exposed to a combination of bFGF and heparin at 0.25 U/ml for 60 h were resistant to the disruptive effects of thrombin on barrier function (Fig. 11). Prolonged exposure to heparin at 0.25 U/ml and bFGF at 10 ng/ml attenuated the amplitude of decline and accelerated the recovery of thrombin-mediated barrier dysfunction. Exposure of cultured monolayers to heparin at >0.25 U/ml in the presence of bFGF did not modify thrombin-mediated barrier dysfunction. Exposure to bFGF alone enhanced the recovery of thrombin-mediated barrier dysfunction but did not attenuate the amplitude of the decline of TER in response to thrombin. Taken together, these data demonstrate that heparin mediates additive effects in enhancement of bFGF-dependent constitutive barrier function and, at low concentrations, further modulates thrombin-mediated barrier function.

Impact of other growth factors and heparin on thrombin-mediated barrier dysfunction. Exposure of cultured monolayers to EGF modified thrombin-mediated barrier dysfunction (Fig. 12). Prolonged exposure of cultured monolayers to EGF attenuated the decline of thrombin-mediated barrier dysfunction compared with control monolayers. Thrombin-mediated barrier dysfunction was not particularly altered in monolayers that had been exposed to VEGF or bFGF for 60 h.

However, a combination of VEGF and heparin further increased the extent of thrombin-mediated barrier dysfunction in cultured monolayers (Fig. 13). Thrombin mediated a greater and more sustained fractional reduction of TER in monolayers exposed to medium containing VEGF and heparin for 60 h than in control monolayers. Again, in monolayers exposed to EGF and heparin for 60 h, thrombin-mediated barrier dysfunction was attenuated compared with control monolayers. Thrombin mediated little difference in TER in bFGF- and heparin-conditioned monolayers compared with control monolayers.
Taken together, these data demonstrate that EGF-dependent conditioning of the endothelium attenuates thrombin-mediated barrier dysfunction independent of heparin, whereas VEGF-dependent conditioning augments thrombin-mediated barrier dysfunction in the presence of heparin.

Impact of growth factors and heparin on endothelial cell membrane properties. Because heparin and growth factors alter constitutive TER, these factors must modulate $C_m$, $R_b$, and/or $\alpha$. Using the numerical model that breaks down the measured TER into $R_b$, $\alpha$, and $C_m$, we further elucidated the mechanism by which heparin and bFGF modulate constitutive endothelial barrier function (Fig. 14). Compared with control monolayers, heparin and bFGF significantly increased $R_b$ and decreased $C_m$. In contrast, no difference was observed in $\alpha$ between heparin- or bFGF-treated monolayers and control monolayers. Taken together, these data demonstrate that bFGF and heparin increase constitutive TER by increasing $R_b$ and decreasing $C_m$. Furthermore, the mechanical phenotype of heparin- and bFGF-treated pHUVEC monolayers was unique compared with that of cHUVEC, despite the fact that all cultured monolayers display high basal TER.

DISCUSSION

This report extensively documents the impact of growth factors and heparin on cultured endothelial cell function under static and dynamic experimental conditions. Mitogens are often added to commercial medium to enhance human endothelial cell proliferation for preclinical drug development. Commercial endothelial cells are also used to elucidate and predict molecular mechanisms of signal transduction in vascular biology. Use of cultured endothelial models in basic and applied research may lead to misleading and complex information if mitogens significantly modulate endothelial function.
Our results document the challenge in interpreting and contrasting published data and building new knowledge without precise knowledge of the physiological phenotype of cultured endothelial cells. In this report, we demonstrate profound quantitative and qualitative differences in endothelial barrier function between cultured pHUVEC and cHUVEC in terms of expressed constitutive and agonist-mediated barrier function. We show the impact of several growth factors and heparin on constitutive and thrombin-mediated endothelial barrier function. The results of these studies have wide implications for using cultured endothelial cells as in vitro models and for contemplating new strategies for use of heparin and growth factor to treat vascular disorders such as inflammatory edema and cancer.

We have demonstrated much lower basal TER in isolated cultured pHUVEC grown in the absence of growth factors than in cHUVEC grown in the presence of growth factors. On the basis of our previously reported numerical model of TER (19, 22, 24), the increased basal TER in cHUVEC was due predominately to disturbances in \( \alpha \) and \( C_m \). These data indicate that culture conditions enriched in growth factors alter endothelial membrane properties, with unique spatial membrane effects.

This report also emphasizes the requirement for differences in the management of numerical modeling of TER to derive measurements of \( \alpha \), \( R_b \), and \( C_m \) between cultured pHUVEC and cHUVEC. There was greater error between \( Z_c \) and \( Z_m \) at high and low current frequencies in cultured cHUVEC, whereas modeling error occurred only at low current frequencies in cultured pHUVEC. Attempts to model the experimental TER at the same frequencies in cultured cHUVEC and pHUVEC would result in greater error and model instability. Thus, in modeling of TER, more reliable and numerical solutions for cytoskeletal membrane properties require minimization of \( \chi^2 \) and \( Z_{\text{error}} \).

We have reported marked differences in thrombin-, cAMP agonist-, and VEGF-mediated endothelial barrier dysfunction, depending on the presence of growth factors. 1) Thrombin mediated a quantitatively greater amplitude and duration of decline in TER in cHUVEC than in pHUVEC. 2) Pretreatment of cultured monolayers with cAMP agonists did not attenuate the decline in TER in thrombin-stimulated cultured pHUVEC, but cAMP stimulation accelerated the restoration of thrombin-mediated barrier dysfunction (20). In contrast, the same cAMP agonists completely prevented the thrombin-mediated decline in endothelial barrier function in cHUVEC. 3) VEGF mediated a slow decline in barrier function in cultured pHUVEC, whereas VEGF induced a rapid but transient decline in barrier function in cHUVEC in serum-free medium. VEGF enhanced barrier function at later time points. Taken together, experimental culture conditions induce artificial but dynamic quantitative and qualitative differences in physiological responses to exogenous stimuli, which demonstrates the need for more knowledge of the variables in cultured conditions that alter outcome measurements. In the case of thrombin stimulation, the data demonstrate quantitative differences in endothelial function. However, the physiological responses of endothelial
function to VEGF and cAMP agonists reflected the strong influence of culture conditions. Consistent with our observation that bFGF, VEGF, and EGF regulate endothelial barrier function, hepatocyte growth factor and keratinocyte growth factor augment endothelial barrier function (9, 11, 16).

We have reported that growth factors directly modulate endothelial barrier function. bFGF enhanced constitutive endothelial barrier function more than any other growth factor tested. In contrast, we did not observe a specific impact of EGF or VEGF on constitutive endothelial barrier function in serum-containing medium. By utilizing a previously published computational algorithm that resolves transendothelial impedance into indexes of cell membrane properties (3), we were able to elucidate that bFGF enhanced TER by increasing $R_b$ and decreasing $C_m$.

Our data also document that individual growth factors antagonize the action of other growth factors on endothelial barrier function. EGF antagonized the enhancing effect of bFGF on constitutive endothelial barrier function. The precise mechanism by which EGF antagonized bFGF is not well understood, nor was it determined in this report.

Growth factors also modulate agonist-mediated barrier function. Conditioning of endothelial monolayers for an extended period to EGF stimulation attenuated thrombin-mediated barrier dysfunction. This effect was observed in the presence or absence of heparin. In contrast, conditioning cultured monolayers to bFGF and VEGF had minimal effect on thrombin-mediated barrier dysfunction in the absence of heparin. The precise mechanisms by which EGF modulates thrombin-mediated barrier function are not well understood. Growth factors could modulate thrombin-mediated barrier dysfunction by altering the density or sensitivity of proteinase-activated receptors. Alternatively, growth factors could regulate second messenger signals, or growth factors could modulate cytoskeletal targets that promote or attenuate endothelial barrier function.

This report has documented that heparin directly modulates constitutive and agonist-mediated barrier function. Heparin enhanced constitutive endothelial barrier function to at least the same level as bFGF. To our knowledge, bFGF and heparin separately and in combination increased TER to the highest reported degree (45–87% above control levels). Similar to bFGF, heparin enhanced endothelial barrier function by increasing $R_b$ and decreasing $C_m$. Conditioning the endothelium at low concentrations of heparin in the presence and absence of bFGF attenuated thrombin-mediated barrier dysfunction. Heparin had no measurable effect on VEGF-mediated endothelial barrier function. However, heparin, in the presence of EGF, decreased endothelial barrier function below control levels, but only at 24 h.

The mechanism by which heparin and growth factors modulate constitutive and agonist-mediated endothelial barrier function remains undefined. Although bFGF and heparin stimulated cultured pHUVEC and cultured CHUVEC expressed high basal TER, there were clear phenotypic differences in endothelial cell membrane parameters. Cultured CHUVEC and pHUVEC displayed predominantly differences in $\alpha$ and $C_m$. In contrast, bFGF and heparin induced their primary effects on $R_b$ and $C_m$ with no remodeling of $\alpha$.

Taken together, these data have important implications, because they suggest potential therapeutic benefits of individual growth factors or a combination of select growth factors and heparin for vascular disorders in which endothelial permeability is an important determinant. Heparin and growth factors could be considered separately or in combination to treat vascular disorders such as inflammatory edema or metastatic vascular dissemination of cancer. Cancer metastasis is major determinant of cancer survival. Previously reported clinical and animal data documented a decrease in metastasis in response to heparin treatment (4, 8). There are several hypotheses for the action of heparin in decreasing metastasis. Heparin and other anticoagulants interfere with tumor cell-platelet association, which is considered a mechanism that facilitates tumor cell adherence and transmigration across the endothelium. It is hypothesized that tumor cell-platelet association stabilizes tumor cell arrest and assists cancer cell survival, protects tumor cells from immune surveillance, and provides tumor cells with critical stimulatory growth factors and cytokines in response to platelet activation (17). However, to our knowledge, a direct benefit of heparin on endothelial barrier function has not been implicated. In this report, we have documented that heparin and bFGF enhance endothelial barrier function, in part, through direct effects on endothelial $R_b$. Thus the mechanism by which these two molecules target the endothelium makes it an attractive therapy to limit tumor transmigration across the endothelium.

Although in vitro models using individual physiological stimuli are routinely used to predict endothelial barrier function under in vivo conditions, our data suggest that measuring single-agent responses may underestimate what is likely a more complicated endothelial physiology under in vivo conditions. Local endothelial barrier integrity is more likely a complex interaction between local and systemic stimuli that could dampen or amplify endothelial permeability to fluid, macromolecules, and immunocompetent molecules. The temporal nature and magnitude of endothelial barrier integrity require a more complete knowledge of the endothelial phenotype and the composition of the local and systemic chemical and mechanical vascular environment.

This report has demonstrated that the standard criteria used to characterize and certify endothelial cells are not sufficient for prediction of in vitro human endothelial function of primary cell lines. Certification of cultured endothelial cells on the basis of cell morphology and biomarkers is not sufficient for prediction of static and dynamic functional properties of endothelial cell lines. This report does not endorse a position against commercial human endothelial cell lines. In fact, one could imagine a situation in which cultured endothelial cells grown in defined medium of growth factors could represent an appropriate model for some experimental situations. However, a more thorough characterization and standardization of endothelial cell function would more precisely contrast and interpret molecular signal transduction data from cultured endothelial cell lines.

In summary, we have reported the impact of growth factor and heparin on endothelial barrier function under static and dynamic physiological conditions. Differential endothelial phenotypes between pHUVEC and CHUVEC are dependent on the growth factors and heparin added to the culture medium. In characterizing the precise impact of several mitogens and heparin on constitutive and thrombin-mediated endothelial barrier function, our data implicate potential new strategies for utilization of growth factors and heparin-like mol-
ecules to treat vascular disorders in which endothelial barrier integrity is a critical determinant, such as tumor vascular metastasis and inflammatory edema. Our data suggest that endothelial barrier integrity may be a complex interaction between local and systemic chemical and mechanical stimuli that dampen or amplify endothelial permeability to fluid, macromolecules, and immunocompetent molecules. A more complete knowledge of the endothelial phenotype and local and systemic chemical and mechanical environment may better predict vascular function. Application of TER represents a quantitative and dynamic measurement that characterizes endothelial phenotype for preclinical drug development and elucidation of vascular molecular signal transduction.

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