Electrical stimulation directs migration, enhances and orients cell division and upregulates the chemokine receptors CXCR4 and CXCR2 in endothelial cells

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Abstract
Natural direct current electric fields (DC EFs) within tissues undergoing angiogenesis have the potential to influence vessel formation, but how they affect endothelial cells is not clear. We therefore quantified behaviours of human umbilical vein endothelial cells (HUVEC) and human microvasculature endothelial cells (HMEC) stimulated by EFs *in vitro*. Both cell types migrated faster and toward the cathode; HUVECs responded to fields as low as 50mV/mm but the HMEC threshold was 100 mV/mm. Mitosis was stimulated at 50 mV/mm for HMEC and at 150 mV/mm for HUVECs, but the cleavage plane was oriented orthogonal to the field vector at 200 mV/mm for both cell types. That different field strengths induced different cell responses suggests distinct underlying cellular mechanisms. A physiological electric field also upregulated expression of CXCR4 and CXCR2 chemokine receptors and upregulated phosphorylation of both chemokines in HUVEC and HMEC cells. Evidence that DC EFs direct endothelial cell migration, proliferation and upregulate chemokines involved in wound healing suggests a key role for electrical control of capillary production during healing. Our data contribute to the molecular mechanisms by which DC EFs direct endothelial cell behaviour and present a novel signalling paradigm in wound healing, tissue regeneration and angiogenesis-related diseases.

Key words: Angiogenesis, wound healing, electric field stimulation, endothelial cells, cell division, chemokine receptor

Introduction

Angiogenesis, the formation of new blood vessels, requires directed migration of endothelial cells [1]. The ability to control it has significant implications for angiogenesis-related clinical conditions. Cells exhibit a variety of responses to applied DC EFs of physiological strengths, including migration toward the cathode (negative pole) or toward the anode (positive pole), with these electrotaxis responses being cell-type and species specific [2,3]. For example, human microvasculature endothelial cell-1 (HMEC-1) cells from angiogenic microvasculature migrate toward the cathode, whilst human umbilical vein endothelial cells (HUVEC) derived from non-angiogenic macrovasculature migrate toward the anode [4]. Although, the mechanisms by which cells sense DC EFs and respond with directional migration are not understood fully [2,3,5–7], these differences in polarity make HMEC and HUVEC cell lines useful to dissect the distinct mechanisms responsible for opposed electrotaxis of angiogenic and non-angiogenic cells.

In the context of cells key to angiogenesis, electric fields of 150 to 400 mV/mm induce physical changes in endothelial cells that include reorientation of the long axis of the cell [8], cell elongation [9], cell proliferation [10], altered cell shape [11] and directional cell migration [8,9,12]; each of these cellular behaviours is essential for angiogenesis. In addition to directing migration, physiological EFs can influence the rate and direction of cell division [13,14] and promote or inhibit cell proliferation, depending on cell type and the electric field strength [15–18]. Although it was previously reported that HUVEC and HMEC cells exhibit opposite electrotaxis responses [9], nothing is known regarding the influence of DC EFs on the axis of HUVEC or HMEC cell division and proliferation.

Two previous studies have shown that electric fields applied *in vitro* upregulate angiogenic factors in starved endothelial cells. DC EFs of 200 mV/mm increased secretion of vascular endothelial growth factor (VEGF) and Interleukin 8 (IL-8) in starved HUVEC cells [4,8]. Additionally, inhibition of VEGF receptors abolished the release of VEGF and IL-8 induced by electric fields, suggesting an autocrine pathway by which an EF activates angiogenic responses [4].

Chemokines are secreted molecules that induce directed cell migration by binding to a receptor, activating several downstream signaling pathways, including phosphatidylinositol 3-kinases (PI3K), Rho-family members, integrins and polarizing the actin cytoskeleton and internalization of the receptors through vesicular transport. Collectively these events influence cell migration and proliferation [19].

Stromal cell-derived factor 1 (SDF-1), the cytokine ligand for the CXCR4 receptor, mediates angiogenesis directly by recruiting endothelial progenitor cells [20], and indirectly, by inducing secretion of pro-angiogenic factors (e.g. VEGF) by endothelial cells that express CXCR4 [21]. CXCR4 is expressed abundantly in HMEC and HUVEC cells [22,23], although it is expressed more strongly in large vessel endothelium than microvessel endothelium [24]. IL-8, the ligand for the CXCR2 receptor is a potent angiogenic factor, with high-affinity binding to the CXCR1 and CXCR2 chemokine receptors [25,26]. Both receptors are present on HUVEC and HMEC cells [23,27–29] but only CXCR2 expression is necessary for endothelial cell chemotaxis [27,29,30]. IL-8 signaling is not only crucial for leukocyte migration but also to stimulate endothelial cell proliferation, permeability, and migration, and to attract lymphocytes, macrophages, and neutrophils to perivascular regions [31]. Direct current electric fields upregulate the expression of some growth factors receptors, including epithelial growth factor receptors on corneal epithelial cells [32]. Whether electric fields also upregulate the expression of chemokine receptors has not been studied. Our results demonstrate upregulation of chemokine receptors by electric fields.

Methods

Cell culture

HUVEC primary human umbilical vein endothelial cells (CC-2517, Lonza) were grown in endothelial basal medium (EBM-2, CC-3156, Lonza) supplemented with 2% FBS, hFGF-β, hydrocortisone, VEGF, R3-IGF-1, ascorbic acid, heparin, hEGF and GA-1000 (SingleQuots supplement, C-4176, Lonza). HMEC primary human blood microvascular endothelial cells (CC-2813, Lonza) were grown in endothelial basal medium (EBM-2, CC-3156, Lonza) supplemented with 5% FBS, hFGF-β, hydrocortisone, VEGF, R3-IGF-1, ascorbic acid, hEGF and GA-1000 (SingleQuots supplement, C-4147, Lonza). Both cell lines were seeded in T75 flasks and grown at 37°C in a humidified incubator with 5% CO_2. The medium was replaced every 2– 3 days and cells were grown to near confluence and passaged when required. Both endothelial cell lines were used until passage 10.

Cell migration and electrotaxis assay

Cell migration was assayed using an electrotaxis apparatus [33,34].
HUVEC and HMEC cells were seeded into prepared chambers coated with 10μg/cm^2 collagen Type I solution from rat tail (C3867, Sigma) at a density of 2x10^4 cells/ml. Cells were allowed to grow for at least 24 h in their respective medium at 37°C in a 5 % CO_2 incubator. After 24h the medium was removed and cells were washed with 5ml of warmed culture medium. The electrotaxis apparatus consisted of a No 1 glass 22x40 mm coverslip 'roof' secured with DC4 silicon compound (Dow Corning) on top of two 11 x 20 mm coverslip spacers placed 1 cm apart to make a central channel through which the electrical field passed (Fig.1). Silicon DC4 barriers were then placed over the top coverslip between the two silicone barriers to ensure that medium from the reservoirs passed through the central channel and not over the top coverslip. The final dimensions of the channel through which the electric field passed was 40 mm x 10 mm x 0.2 mm. Finally, 4 ml of culture medium buffered with 25 mM HEPES was added to each side of the chamber. For electric field application, 10 cm long glass tubes (TWL-611-010M, Fisher) were heated to create U-shaped bridges and 2% agar was prepared in boiling Steinberg's solution (58 mM NaCl, 0.67 mM KCl, 0.44 mM $Ca(NO_3)_2$, 1.3 mM $MgSO_4$, 4.6 mM Trizma base, pH 7.8–8.0). The hot agar solution was used to fill the glass tubes to connect the electrical power supply via silver/silver chloride electrodes and Steinberg's solution in beakers to the cells in the electrotaxis chamber [33]. The use of agar salt bridges prevents the diffusion of electrode products into the culture medium and isolates cells from pH changes at the electrodes [3]. Cells were exposed to an electric field in the physiological range of 50 mV/mm to 300mV/mm [8,9] for 3 h at 37 °C in a temperature-controlled chamber on an inverted microscope stage. Serial time-lapse images were recorded using a Nikon ECLIPSE TE2000-U microscope and Simple PCI software (Hamamatsu Corporation, PA, USA).

Quantification of cell migration

Images were captured every 10 minutes from 10 visual fields using the 20X objective, starting when electrical field stimulation started (T=0). Quantification of cell migration was based on two parameters; migration speed, and migration directedness. Migration speed is the total length of the trajectory a cell migrated divided by the time, and migration directedness is based on cosine θ [35] where θ is the angle between the electric field vector and a straight line connecting the start and end position of a cell. A cell moving directly along the field lines toward the anode would have a θ angle of 0 deg and a directedness of 1; a cell moving directly toward the cathode would have a θ angle of 180 deg and a directedness of -1. An average directedness value close to 0 for a population describes random cell movement. The two parameters were measured by tracing the position of cell nuclei before and after electric

field application with Image J software (Manual Tracking plug-in, NIH) and only single cells were analysed.

Quantification of cell proliferation
From the cell migration images the total number of cells was counted as well as the number of cells that divided to create two cells. The percentage of cells dividing was calculated for conditions with an electric field and without an electric field.

Quantification of cell cleavage plane orientation
Quantification of cell orientation with respect to the electric field was defined as cos 2θ, where θ is the angle measured between the two nuclei resulting from phase contrast images of live dividing cells, using the angle tool in Image J software [36]. The significance of an orthogonal /parallel orientation distribution against randomness was calculated using Rayleigh's distribution [35,37]. After collecting the angles of dividing cells Rayleigh's distribution was applied to give an average polarization index (PI) of $(\sum n \cos[2(\theta-90)]/n)$, where n is the number of measurements and θ is the angle between the cleavage plane and the electric field vector. This will give a polarization index that varies from −1 to 1. A cell with its plane of cleavage parallel to the electric field vector will have a polarization of −1, and a cell that divided with a cleavage plane exactly orthogonal to the electric field vector will have a polarization of 1. Cells dividing at random angles will have a population average polarization of 0. Cells dividing with cleavage planes, on average, orthogonal to the electric field vector will have a polarization value between 0 and 1; the higher the value, the more orthogonal the plane of division was to the applied electric field. Cells dividing with cleavage planes, on average, parallel to the electric field vector will have a polarization value between 0 and −1, with −1 indicating all cells with a cleavage exactly parallel to the electric field.

Western Blotting
Protein was extract from HUVEC and HMEC cells that were either grown in complete medium or starved for 24 h in serum-free culture medium before exposure to a 300 mV/mm direct current electric field in vitro. After electric field stimulation cells were rinsed with cold PBS and lysed with lysis buffer CelLytic-M (C2978; Sigma) containing protease inhibitors (11836170001, complete mini EDTA-free, Roche) and phosphatase inhibitors (4906837001, PhosSTOP, Roche). Ten micrograms of total protein were electrophoresed by 12% SDS-PAGE at 100 Volts for 2.5 h at room temperature, and transferred onto nitrocellulose membrane (Z613630, Sigma) using the Bio-Rad system at 100 Volts for 1.5h at 4ºC. Transfer efficiency was assessed by staining membranes briefly with Ponceau S solution and then membranes were blocked with blocking buffer (WBAVDFL01, Millipore) diluted 1x in TBS (20 mM Tris, 150 mM NaCl, pH 7.6) for 1h followed by primary antibody (1:2000 anti-human CXCR4, ab2074; 1:100 anti-human CXCR2, ab21641; 1:1000 phospho-CXCR4, ab74012; 1:1000 phospho-CXCR2, ab61100; 1:20000 anti-GAPDH, ab8245; all antibodies were from Abcam) diluted in blocking buffer (WBAVDFL01, Millipore) overnight at 4°C with slow shaking. Membranes were then incubated with Alexa fluor 790 donkey anti-rabbit (1:2000, Life technologies) and Alexa fluor 680 donkey anti-mouse (1:2000, Life technologies) diluted in TBS-T (Tris-buffered saline, 0.1% Tween 20) for 1h at room temperature. Blots were imaged using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE) and analyzed by measuring integrated intensities in the Image Studio Lite software version 4.0. Results are from three representative Western blots with normalized densitometric arbitrary units.

Statistical Analysis

The results were analysed using a two-tailed Student's t test and one-way analysis of variance (ANOVA) to estimate the probability of the differences. For the analysis of three or more groups of data, Bonferroni or Dunns post hoc tests were used when the P-value (by ANOVA) indicated a statistically significant difference among groups. All tests were performed with confidence intervals set at 95%. Results are presented as the means ± SEM. $P<0.05$ was considered to indicate a statistically significant difference. All statistical tests were performed using GraphPad Prism® 5 (Version 5.04).

Results

Electrotaxis of endothelial cells is faster and is directed to the cathode

Electrotaxis behaviour was quantified for HMEC cells exposed to direct current electric fields ranging from 50 mV/mm to 300 mV/mm and for control conditions without a field (Movie, Supplemental Material). In the absence of an electric field cells migrated at a speed of 13.9 ± 0.06 μm/h (n=3; 1800 cells) (Fig.2 A) and with increasing electric field strength the migration speed increased to 17.8 ± 1.1 μm/h at 100mV/mm (n=3; 300 cells, $P<0.05$) and to 30 ± 1 μm/h at 300 mV/mm, more than double the control speed (n=3; 300 cells, $P<0.05$). HMEC cells were directed toward the cathode at electric fields ranging from100 mV/mm to 300 mV/mm but not at 50 mV/mm (Fig.2 B). Above 50 mV/mm the directional migration increased as the electric field increased, peaking at 300 mV/mm, where directedness was -0.76 ± 0.02 (n=3; 300 cells; $P<0.001$) compared to the control value of -0.03 ± 0.02 (n=3; 1800 cells).

HUVEC cells migrated at a speed of 16.0 ± 0.3 μm/h (n=15; 1500 cells, Fig.3 A) in the absence of an electric field. The migration speed was increased, even at 50mV/mm, the lowest electric field tested, reaching 22.3 ± 2.6 μm/h (n=3; 300 cells, $P<0.001$) . At 300 mV/mm the migration speed increased further to 33.0 ± 1.8 μm/h, more than double that of the control (n=3; 300 cells, $P<0.001$, Fig.3 A). HUVECs migrated toward the cathode in electric fields of 50 to 300 mV/mm. A significant directional migration response was detected even at 50 mV/mm (Fig.3 B), indicating that the threshold for directional migration must be even lower than this. Directional migration increased in a linear manner and at a field strength of 300 mV/mm directedness reached -0.63 ± 0.02 (n=3; 300 cells; $P<0.001$) compared with the control value of -0.02 ± 0.03, n=15; 1500 cells).

The substratum coating did not affect HUVEC electrotaxis toward the cathode

HUVECs cultured on collagen migrated toward the cathode, opposite to the anodal migration reported previously for HUVECs cultured on collagen or fibronectin [9], so we tested whether a change of substrate would influence the migration direction. This was done at an electric field of 150 mV/mm using substrates coated with the extracellular matrix molecules fibronectin and laminin (Fig.4 A). With no coating HUVEC cells stimulated with the electric field had a migration directedness of -0.46 ± 0.01 (n=2; 200 cells; $P<0.01$) compared to the control value of -0.08 ± 0.02 (n=2; 200 cells). On a collagen substrate HUVECs in an electric field migrated cathodally, with a directedness of -0.42 ± 0.05 (n=2; 200 cells; $P<0.05$) compared with the control value of 0.07 ± 0.02 (n=2, 200 cells). On fibronectin HUVECs in the electric field migrated to the cathode with a directedness of -0.46 ± 0.04 (n=2; 200 cells; $P<0.05$) compared with the control (-0.07 ± 0.02; n=2; 200 cells). Therefore, quantitatively, HUVEC cell electrotaxis at 150 mV/mm was the same on uncoated tissue culture plastic as it was on collagen or fibronectin coated dishes.

The passage number did not affect HUVEC electrotaxis toward the cathode

We also examined whether the cell passage number affected the migration directedness due to potential loss of receptors that control directional migration. HUVEC cells at passages P5, P8 or P10 were exposed to 150 mV/mm and electrotaxis was quantified for 100 cells at each condition and compared to controls at the same passage. Unlike a previous study that used HUVECs at P10 [9] we found that passage number had no effect on directed electrotaxis

(Fig.4 B). P5 HUVECs migrated toward the cathode, with a directedness of -0.47±0.06 ($P< 0.001$) compared to control value of 0.04±0.07. At P8 HUVECs migrated cathodally with a directedness of -0.47±0.06 ($P< 0.05$) compared with the control value of -0.03±0.07 and at P10 HUVECs also migrated toward the cathode with a directedness of -0.46±0.06 ($P< 0.05$) compared with the control value of -0.06±0.07.

Electric fields stimulate endothelial cell proliferation and orientate the cleavage plane

The effect of applied electric fields on endothelial cell proliferation and on the plane of cell division have not yet been described. However, in epithelial cells endogenous electric fields increased the frequency of cell division [14] and dividing cells showed cleavage plane alignment orthogonal to the electric field vector [13,14]. In the present study, the percentage of HUVEC cells undergoing mitosis increased significantly at 150 mV/mm and at 300 mV/mm but at 50 mV/mm the increase was not significant (Fig.5 A). On the other hand, the percentage of dividing HMEC cells increased significantly over the range of electric fields tested (50 mV/mm to 300 mV/mm) although the increase at 150 mV/mm did not reach statistical significance (Fig.5 B). Therefore, cell division was stimulated in both cell types, but in HMECs cell division was stimulated at a lower electric field threshold than in HUVEC cells.

The cleavage plane of cells with no electric field stimulation was oriented randomly, but in cells exposed to a physiological electrical fields the cleavage plane was largely orthogonal (at right angles) to the field vector (Fig.6). In comparison to controls the cleavage plane of dividing HUVEC cells was more frequently oriented orthogonal to the electric field vector at 200 mV/mm, but at 100 mV/mm this orientation shift was not significant (Fig.7 A). Control cells with no field had a polarization value of 0.04 ± 0.04 (n=365 cells), indicating random cleavage plane orientation and cells dividing in an electric field of 200 mV/mm had a polarization value of 0.25 ± 0.01 (n=164 cells), indicating an orthogonal bias in cleavage orientation. There was a tendency to orthogonal orientation of the cleavage plane in HMEC exposed to fields ranging from 50 mV/mm to 300 mV/mm, but this was only statistically significant at 200 mV/mm (Fig.7 B). Dividing control cells (no field) had a polarization value of 0.001 ± 0.04 (n=267), indicating random cleavage orientation, whereas cells exposed to 200 mV/mm had a polarization value of 0.36±0.04 (n=117), indicating a bias toward orthogonal cleavage plane orientation.

Quantification of the total protein levels of CXCR4 and CXCR2 in HUVEC and HMEC whole cell lysate

To analyze roles of the chemokine receptors CXCR2 and CXCR4 potentially in directing endothelial cell migration we first compared the levels of total CXCR4 and CXCR2 between HMEC and HUVEC (Fig.8). HMEC had similar levels of CXCR4 and CXCR2 while HUVEC showed higher levels of CXCR4 compared to CXCR2. Between HUVEC and HMEC, HUVEC showed higher levels of both CXCR4 and CXCR2 compared to HMEC. Therefore, both chemokine receptors are expressed in both endothelial cell types.

Quantification of CXCR4 and CXCR2 during electric field exposure

HMEC and HUVEC cells migrated toward the cathode at different electric field strengths (Fig 2 and Fig 3) and the chemokine receptors CXCR4 and CXCR2 are involved in endothelial cell migration [38,39] so we explored the relative roles of CXCR4 and CXCR2 in migration of HUVEC and HMEC cells by quantifying their expression during electric field exposure. In HMECs the levels of total CXCR4 protein relative to no field controls were increased at 15 min and at 30 min of electric field stimulation and then decreased significantly by 1h (Fig.9 A). In HUVEC cells the level of total CXCR4 compared to controls also increased by 15min and 30min of electric field exposure but at 1h it remained elevated (Fig.9 C). The total CXCR2 protein levels in HMECs were increased by 15 min and 30 min, increasing further by 1 h (Fig.9 B). In HUVEC cells the levels of CXCR2 gradually increased from 15 min to 30 min and then decreased slightly at 1h but CXCR2 levels did not change during electric field exposure (Fig.9 D). In terms of maximum increase of the levels of total CXCR4 and CXCR2 with electric field exposure it was observed that in HMEC cells CXCR4 have maximum increase at 15 min and CXCR2 at 1 h while HUVEC showed maximum increase of CXCR4 at 1 h and CXCR2 at 30 min.

Quantification of CXCR4 and CXCR2 phosphorylation during electric field exposure

In HMECs electric field stimulation increased the levels of both phospho-CXCR4 and phospho-CXCR2 at 15 min, 30 min and 1 h compared to controls (Fig.10 A, Fig.10 B). In HUVECs the levels of phospho-CXCR4 increased at 15 min, 30 min and 1 h compared to controls (Fig.10 C), whereas phospho-CXCR2 levels increased at 15 min and 30 min during electric field stimulation compared to controls (Fig.10 D). Between 15-30 min the electric field increased the levels of phospho-CXCR2 but by 1 h the levels of phospho-CXCR2 had decreased (Fig.10 D).

Discussion

During wound healing and tumour formation, situations where DC EFs exist naturally, vascular endothelial cells migrate directionally to form new blood vessels [1,40,41]. It is known that applied DC EFs induce directional migration of endothelial cells and that the electric fields accelerate their migration speed [4,8,9]. However, nothing is known about the effect of DC EFs on the orientation and frequency of endothelial cell division, which also are key events during angiogenesis. We show that HUVECs migrated toward the cathode in electric fields ranging from 50 mV/mm to 300 mV/mm (Fig.3 B), opposite to the anodal migration reported previously [9]. The reason for this difference is not clear, but at 200 mV/mm we found a robust cathodal response with a directedness of -0.55 ± 0.03 ($n = 300$) compared to their reported very slight anodal response of 0.15 ± 0.08 ($n = 66$) [9]. We explored this further by testing whether the passage number of HUVEC cultures or the substratum might have contributed to the variability. We found remarkably consistent cathodal migration for cultures ranging from P5 to P10 (Fig.4 B), so excluding passage number as a contributory factor (Bai et al. used only P10 HUVECs). The substratum coating also can affect cell responses to direct current electric fields, with increased cathodal responsiveness on extracellular matrix proteins such as fibronectin or laminin [32] and the reversal of cathodal nerve outgrowth on plastic or laminin to anode directed growth on polylysine [42]. Here HUVECs showed strong, consistent migration toward the cathode on plastic, collagen and fibronectin (Fig.4 A). It remains possible that there were subtle differences in the cell lines used for these two studies because Bai et al., [9] sourced HUVECs from American Type Culture Collection and ours were from Lonza.

Although HMECs and HUVECs exhibited electrotaxis to the cathode they showed subtle differences in their responses to electric fields. HUVECs were more responsive at lower electric fields, with a response threshold for cathode-directed migration and increased migration speed at 50 mV/mm, the lowest electric field tested, whereas HMECs only started to respond at a threshold somewhere between 50 and 100 mV/mm. That HUVEC and HMEC cells can be directed by electric fields as low as 100 mV/mm suggests an important role of external electric field stimulation as a tool for angiogenesis during wound healing. Electric fields of 40 to 100 mV/mm are present in normal healing wounds and are important in controlling the orientation and frequency of cell division [14] and directional migration toward the injury [43]. During wound healing, new capillaries grow in a directed manner into the wound site, so our data suggest that electric fields may promote wound healing by directing endothelial cells to the wound site to initiate angiogenesis.

Cell proliferation is controlled at the G1/S cell cycle checkpoint, the transition from the first gap phase (G1) phase to the DNA synthesis phase (S). Activation of cyclin proteins control proliferation during the G1 phase [44]. Cyclin D1 plays a central role in the regulation of proliferation and is required for progression through the G1 phase [45]. External physical forces (e.g., shear stress) increase cyclin D1 and downregulate the cell cycle inhibitor p21 in vascular smooth muscle, promoting proliferation [46], so other physical external factors (direct current electric fields) also may affect endothelial proliferation. A DC EF of 200 mV/mm decreased the expression of Cyclin E in vascular endothelial cells, preventing passage through G1, whilst increasing the expression of $p27^{kip1}$, an inhibitor of the cyclin E/Cdk2 complex [10]. The decrease of cell division observed in HUVECs exposed to 200 mV/mm (Fig 5) may therefore be related to lower expression of Cyclin E. This remains to be tested in HUVEC and HMEC cells.

In dividing HMEC and HUVECs the mitotic cleavage orientation was orthogonal to the 200 mV/mm electric field vector. This is consistent with reports for dividing epithelial cells at 150 mV/mm [13] and for cleavage orientation of corneal epithelial cells in the rat eye under the influence of an endogenous wound-induced electric field [47]. The cleavage plane, which positions the daughter cells relative to each other, is regulated by the interaction of the mitotic spindle with the cortical actin cytoskeleton [48,49]. Endothelial cells orient their actin cytoskeleton and microtubule network in response to shear stress, as would be produced by blood flow *in vivo* [50]. It has been suggested that an electric field regulates the axis of cell division by orienting the mitotic spindle [14], which becomes aligned parallel to the electric field vector leading to an orthogonal plane of cell division [13]. In a flow-independent model of dynamic angiogenesis in culture and in retinal vessels *in vivo*, endothelial cell cleavage was oriented orthogonal to the long axis of the vessel, which may promote vessel lengthening [48]. Interestingly, blood flow itself induces electrical signals called streaming potentials in microvessels, which at the endothelial cell surface are between 1 – 3 mV/mm [51]. Depending on the strength of the electric field different responses were observed in endothelial cells. At 200 mV/mm cleavage orientation was seen for both HUVEC and HMECs; at 50 mV/mm cell mitosis frequency was influenced in both endothelial cell lines and 50 mV/mm also influenced migration direction of HUVECs. Mechanistically, the differences in cell responses to a uniform electric field suggest that the mechanisms underpinning directed electrotaxis, migration speed and cell division must be distinct from those controlling the orientation of the mitotic spindle and proliferation rate. A molecular mechanism for elongation, orientation and migration of endothelial cells has been proposed that points to the involvement of VEGF receptors as the proximal element to transduce the electric field signals. This then mediates pro-angiogenic responses through downstream signals involving PI3K-Akt, Rho-ROCK and the F-actin cytoskeleton [8,52].

Several aspects of these findings require clarification. Firstly, the use of EFs of 50 – 100mV/mm is physiological. Bio-potentials have been measured across human and guinea pig skin and across bovine cornea. These can vary from around 50 – 200mV. When these tissues are wounded, these normal potentials give rise instantaneously to electrical fields at the wound edge which are in the order of 50 – 100mV/mm [53–56]. EFs of this size have been shown to direct epithelial cell migration and division, enhance nerve sprouting and growth into the wound and stimulate macrophage directed invasion and phagocytic activity [3,57,58].

In most instances, these electrical gradients within the extracellular spaces are generated by differential pumping of ions across epithelial, but also across endothelial sheets of cells. This establishes a trans-epithelial/endothelial potential difference (TEPD) that varies both spatially and temporally to generate endogenous electrical fields. Wounding such a system short circuits the TEPD, giving rise to a steady electrical gradient directed towards the wound as ionic currents leave through the lesioned area. These concepts are outlined more thoroughly in two reviews [3,57]. Although ionic gradients can be induced in the extracellular spaces by EFs *in vivo*, previous *in vitro* studies using an EF chamber set up similar to that used here proved that cellular responses to EFs persist in the presence of continuous fluid flow during EF exposure [9]. Such flow would disrupt any ionic gradients established by the EF, indicating that the voltage gradient is the dominant cue, at least in vitro.

There are several other examples of injury associated electrical signals. In breast cancer, for example, potential differences between proliferating and non-proliferating regions can be

measured at the surface of the skin and are used diagnostically because they correlate well with malignancy of the neoplasm [59,60].

There are also several types of electric potential difference around the endothelium of blood vessels that might be involved in regulating the development of thromboses. The ζ potentials, for example, are created at the endothelial cell wall by the flow of blood in both the aorta and vena cava, and range from 100 mV to 400 mV [61,62].

Importantly, in the presence of an EF of 100 mV/mm, an endothelial cell 50 μm long would experience a voltage drop of around 5 mV along its length. This is enough to synchronously drive beating heart cell contractions [63]. Although the endothelium is non-contractile this cardiac cell example emphasizes the fact that these small electrical gradients can stimulate normal physiological functions. Although unlikely to induce long range ionic gradient redistribution, similarly sized electrical fields in the developing limb bud influence the distribution of charged protein molecules in the extracellular space [64].

The expression of chemokine receptors by endothelial cells has been controversial [27,65,66]. We detected the chemokine receptors CXCR4 and CXCR2 in both endothelial cell lines, consistent with other studies [22,23,29,67,68]. In whole cell lysates HUVECs expressed more CXCR4 and CXCR2 receptors than HMECs. Comparing both chemokines, HUVEC expressed more CXCR4 than CXCR2 while in HMEC the expression of both chemokines was similar (Fig.8). Flow cytometry studies showed that CXCR4 and CXCR2 were expressed more in large vessel endothelium than microvessel endothelium [24], but confocal microscopy and immunofluorescence indicated that HUVEC and HMECs expressed similar levels of CXCR4 and that HMECs expressed more CXCR4 than CXCR2 [23]. The different results might be related to the heterogeneity amongst HUVEC primary cultures as well as differences among batches and cell culture conditions [66].

Increasing time of electric field exposure affected the expression of total CXCR4 and CXCR2 in the cell lines differently. While in HMEC the levels of CXCR4 increases and then fell at 1h and the levels of CXCR2 increases with increasing electric field exposure in HUVEC the pattern is contrary (levels of CXCR4 increases with increasing time of electric field exposure and the levels of CXCR2 increases and then falls slightly at 1h). In neutrophils high changes in levels of CXCR4 and CXCR2 trigger their migration from the bone marrow [69]. HMEC cells after 1h exposed to electric fields showed a decrease on the levels of CXCR4 and an increase on the levels of CXCR2, suggesting that 1h of electric field stimulation could trigger endothelial migration from microvasculature toward a wound.

Finally, our analyses show that electrical gradients induce both receptor protein expression and receptor protein phosphorylation rapidly, within 5 – 10 minutes. The mechanisms underpinning these rapid changes are unclear but have been observed in stressed yeast cells and in human microvascular cells where hypoxia rapidly enhanced CXCR4 expression [70,71].

Figure 11 shows a hypothetical mechanism for electric field induced angiogenic responses of endothelial cells. Electric field stimulation induces the release of pro-angiogenic factors such

as VEGF and IL-8 and upregulate the expression of chemokine receptors CXCR4 and CXCR2. The interaction of SDF-1 with CXCR4 amplifies angiogenesis by inducing VEGF release. VEGF induces CXCR4 and SDF-1 production by endothelial cells, creating a positive-feedback loop. VEGF release stimulated by electric field binds to VEGFR activating important intracellular signaling pathways like PI3K/Akt and Rho/ROCK signalling pathways, resulting in directional organization of the cytoskeleton, cell elongation, alignment and directional cell migration.

Conclusions

Electrical stimulation in wound healing has been demonstrated by its ability to induce the re-epithelialization of cutaneous and corneal wounds through promoting migration and proliferation of fibroblasts, keratinocytes, epithelial cells and endothelial cells enhancing angiogenesis, improving blood circulation, and blocking edema formation [14,34,36,72–75]. Regarding endothelial cells, electric fields directed HUVEC and HMEC cells toward the cathode, increased cell proliferation and oriented the axis of cell division orthogonal to the electric field vector. In addition to promoting migration, electric fields increased expression of the chemokines CXCR4 and CXCR2. Furthermore, electric field stimulation increases the secretion of IL-8, a cytokine known to play an important role in wound repair and promoting the growth of new blood vessels. Overall, electric fields give endothelial cells a powerful directional cue to guide cell migration, elongation, and alignment, which are important angiogenic responses that may lead to organized vessel formation. The advancement in technology of application of electrical stimulation and improved understanding of biological effects of such stimulation will lead to new therapies to enhance repair and regeneration of blood vessels and to treat diseases or conditions in which angiogenesis is abnormal.

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