DC Electric Fields Induce Distinct Preangiogenic Responses in Microvascular and Macrovascular Cells

Huai Bai, Colin D. McCaig, John V. Forrester, Min Zhao

Objective—Electrical stimulation induces significant angiogenesis in vivo. We have shown recently that electrical stimulation induces directional migration, reorientation, and elongation of macrovascular endothelial cells. Because angiogenesis occurs mainly in the microvasculature, we have extended this observation to include human microvascular endothelial cells (HMEC-1s) and compared the responses with that of vascular fibroblasts and smooth muscle cells and human umbilical vein endothelial cells.

Methods and Results—Four types of vascular cells were cultured in electric fields (EFs). Dynamic cell behaviors were recorded and analyzed with an image analyzer. EFs of 150 to 400 mV/mm induced directed migration, reorientation, and elongation of all the vascular cells. HMEC-1s showed the greatest directional migration (migration rate of 11 μm/h and directedness of 0.35 at 200 mV/mm). Most intriguingly, HMEC-1s migrated toward the cathode, whereas the other cell types migrated toward the anode.

Conclusions—Endothelial cells derived from angiogenic microvascular as opposed to nonangiogenic macrovascular tissues were more responsive to electrical stimulation. This intriguing directional selectivity indicates that a DC electrical signal as a directional cue may be able to play a role in the spatial organization of vascular structure.

Key Words: vascular cells ■ electrical stimulation ■ angiogenesis ■ heterogeneity ■ cell migration ■ alignment ■ orientation
increase growth factor release.\(^2,28,29\) These changes are important for migration of many cell types, including human umbilical vein endothelial cells (HUVECs) and bovine aortic endothelial cells.\(^5,7,8\) Because angiogenesis occurs mainly in the microvasculature usually at post-capillary venules and not in large blood vessels,\(^30\) we studied the effects of a DC EF on an HMEC-1 line.\(^31\) In addition, because the vascular wall contains different endothelial and other cell types that are likely to be exposed to the same EF in vivo or when EFs are applied exogenously, we compared the responses of HMEC-1s with those of vascular fibroblasts, vascular smooth muscle cells (SMCs), and the previously studied HUVECs. We show that endothelial cells derived from angiogenic microvasculature as opposed to macrovascular tissues moved fastest in the opposite direction in a small DC EF. Thus, different cell types from a common tissue source responded differently to an applied DC EF. This intriguing directional selectivity indicates that a DC electric signal as a directional cue may be able to play a role in the spatial organization of vascular structure.

**Methods**

**Reagents and Cell Culture**

DMEM, FBS, and other cell culture reagents were from Gibco/BRL. HMEC-1s (from Dr. F.J. Candal, Centers for Disease Control, Bethesda, Md) were cultured as described previously in growth factor complete endothelial basic medium (EBM; Clonetics) supplemented with FBS (10%),\(^31\) Murine aorta SMC (MASMC) line (from Dr. F.J. Candal, Centers for Disease Control, Bethesda, Md) were cultured as described previously in growth factor complete endothelial basic medium (EBM; Clonetics) supplemented with FBS (10%).\(^31\) Murine aorta SMC (MASMC) line (from Dr. F.J. Candal, Centers for Disease Control, Bethesda, Md) were cultured as described previously in growth factor complete endothelial basic medium (EBM; Clonetics) supplemented with FBS (10%).\(^31\) Murine aorta SMC (MASMC) line (from Dr. F.J. Candal, Centers for Disease Control, Bethesda, Md) were cultured as described previously in growth factor complete endothelial basic medium (EBM; Clonetics) supplemented with FBS (10%).\(^31\) Murine aorta SMC (MASMC) line (from Dr. F.J. Candal, Centers for Disease Control, Bethesda, Md) were cultured as described previously in growth factor complete endothelial basic medium (EBM; Clonetics) supplemented with FBS (10%).\(^31\) Murine aorta SMC (MASMC) line (from Dr. F.J. Candal, Centers for Disease Control, Bethesda, Md) were cultured as described previously in growth factor complete endothelial basic medium (EBM; Clonetics) supplemented with FBS (10%).\(^31\) Murine aorta SMC (MASMC) line (from Dr. F.J. Candal, Centers for Disease Control, Bethesda, Md) were cultured as described previously in growth factor complete endothelial basic medium (EBM; Clonetics) supplemented with FBS (10%).\(^31\) Murine aorta SMC (MASMC) line (from Dr. F.J. Candal, Centers for Disease Control, Bethesda, Md) were cultured as described previously in growth factor complete endothelial basic medium (EBM; Clonetics) supplemented with FBS (10%).\(^31\) Murine aorta SMC (MASMC) line (from Dr. F.J. Candal, Centers for Disease Control, Bethesda, Md) were cultured as described previously in growth factor complete endothelial basic medium (EBM; Clonetics) supplemented with FBS (10%).\(^31\) Murine aorta SMC (MASMC) line (from Dr. F.J. Candal, Centers for Disease Control, Bethesda, Md) were cultured as described previously in growth factor complete endothelial basic medium (EBM; Clonetics) supplemented with FBS (10%).\(^31\) Murine aorta SMC (MASMC) line (from Dr. F.J. Candal, Centers for Disease Control, Bethesda, Md) were cultured as described previously in growth factor complete endothelial basic medium (EBM; Clonetics) supplemented with FBS (10%).\(^31\) Murine aorta SMC (MASMC) line (from Dr. F.J. Candal, Centers for Disease Control, Bethesda, Md) were cultured as described previously in growth factor complete endothelial basic medium (EBM; Clonetics) supplemented with FBS (10%).\(^31\) Murine aorta SMC (MASMC) line (from Dr. F.J. Candal, Centers for Disease Control, Bethesda, Md) were cultured as described previously in growth factor complete endothelial basic medium (EBM; Clonetics) supplemented with FBS (10%).\(^31\) Murine aorta SMC (MASMC) line (from Dr. F.J. Candal, Centers for Disease Control, Bethesda, Md) were cultured as described previously in growth factor complete endothelial basic medium (EBM; Clonetics) supplemented with FBS (10%).\(^31\) Murine aorta SMC (MASMC) line (from Dr. F.J. Candal, Centers for Disease Control, Bethesda, Md) were cultured as described previously in growth factor complete endothelial basic medium (EBM; Clonetics) supplemented with FBS (10%).\(^31\) Murine aorta SMC (MASMC) line (from Dr. F.J. Candal, Centers for Disease Control, Bethesda, Md) were cultured as described previously in growth factor complete endothelial basic medium (EBM; Clonetics) supplemented with FBS (10%).\(^31\) Murine aorta SMC (MASMC) line (from Dr. F.J. Candal, Centers for Disease Control, Bethesda, Md) were cultured as described previously in growth factor complete endothelial basic medium (EBM; Clonetics) supplemented with FBS (10%).\(^31\) Murine aorta SMC (MASMC) line (from Dr. F.J. Candal, Centers for Disease Control, Bethesda, Md) were cultured as described previously in growth factor complete endothelial basic medium (EBM; Clonetics) supplemented with FBS (10%)

**EF Stimulation**

The cell culture, experimental set-up, and field exposure were similar to those reported previously.\(^33\)

**Cell Behavior Quantification**

Serial pictures were taken immediately before EF application and then hourly for up to 5 hours to quantify migration, or at 4, 8, and 24 hours to quantify orientation and elongation as described in detail previously.\(^7,33\) Individual frames were recorded and analyzed using an image analyzer (Q500MC; Leica). All single visually viable cells were analyzed except those that merged into cell sheets or made contact with a reference scratch mark during EF exposure.

**Directional Migration**

Mean migration rate and directedness were quantified during a 5-hour period.\(^33,34\) The migration rate was defined as D/t, where D is the distance of a straight line connecting starting and end position of a cell over this period of time, and t is the duration of time. The angle (θ) that each cell moved with respect to the imposed EF vector was measured. The cosine of this angle (defined as directedness) is 1 for cells moving directly toward the cathode, 0 for cells moving perpendicular to the EF vector, and −1 for cells moving directly toward the anode. Averaging the cosines (Σcosθ/n, where θ is the angle between the field vector and the direction of movement for an individual cell measured in the group of cells, and n is the total number of cells) yields average directedness of cell movement.

**Perpendicular Orientation**

Cell orientation was quantified as an orientation index (Oi).\(^7,33\) Oi of a cell with respect to the EF was defined as a function of cos(2α), where α is the angle formed by the long axis of each cell with a line drawn perpendicular to the field vector, which was measured using the image analyzer. This Oi varies from −1 to 1. A cell lying parallel to the EF vector has an Oi of −1, and a cell perpendicular to the EF vector has an Oi of 1 (Figure 4i). Average Oi for a cell population [Σcos(2α)/n] was calculated, where α is the angle formed by the long axis of a cell with a line drawn perpendicular to the field vector in the group of cells, and n is the total number of cells. A population of cells with each cell oriented in random direction would give an Oi value of 0, whereas with more and more cells aligned more and more perpendicular to EF vector, the Oi value would increase and approach 1. The significance of this orientation was calculated using Rayleigh distribution.\(^33\) The probability that the population is randomly oriented is given by \(P = e^{-\alpha^2/2}\), where \(L = [\Sigma \sin(2\alpha)]^2 + [\Sigma \cos(2\alpha)]^2/n\) (0.01), and n is the total number of cells. A probability level of 0.001 was used as the limit for significant perpendicular orientation.

**Cell Elongation: Long:Short Axis Ratio**

The distance between the 2 points on the edge that are farthest apart and the greatest width measured perpendicular to that line are defined as the long and short axis of a cell. Long and short axes were traced manually with the interactive software of the image analyzer for those cells with distinct long and short axes (Figure 4). A long:short axis ratio was calculated and gives an objective assessment of elongation of the cells tested.

Statistical analysis was made using unpaired, 2-tailed Student t test or Welch unpaired t test when SDs were significantly different from each other. Data are expressed as mean±SEM.

**Results**

**Microvascular and Macrovascular Cells Migrate in Different Direction in EFs**

When cultured without EFs, all 4 types of vascular cells migrated in random directions, although MASMCs moved very little. When cultured in a physiological EF, the 4 types of...
of cells showed evident directional migration. Strikingly, HMEC-1s migrated toward the cathode, whereas BPAFs, MASMCs, and HUVECs migrated anodally (Figure 1a through 1d). HMEC-1s and BPAF cells were the most actively migrating cells in our experimental conditions, with directional migration obvious within 3 hours (Figure 1a and 1b). Cells extended lamellipodia in the direction of migration within 1 hour after the onset of the EF. The size and shape of HMEC-1s changed more frequently than BPAF cells in EFs. MASMCs and HUVECs migrated more slowly, although as shown in Figure 1c, MASMCs did migrate toward the anode very slowly (3.2 μm/h).

The difference in the migration direction in EFs among the 4 types of vascular cells was confirmed and quantified with detailed analysis of the time lapse images at different EF strengths (Figures 2, 3a, and 3c). As described in Methods, cells moving toward the cathode (left) have directedness values approaching 1, whereas cells moving toward the anode have a directedness approaching −1. In an EF of 150 mV/mm, HMEC-1s showed significant cathodal directedness, whereas BPAF cells, MASMCs, and HUVECs migrated anodally (Figures 2, 3a, and 3c). The difference in polarity of directed movement was striking (Figure 3a and 3c).

The vascular cells in our culture conditions migrate slowly compared with another report. To confirm the directional difference in migratory responses of different vascular cells to DC EFs, several different culture media and substratum coating conditions were tested. Although substratum coating of fibronectin or collagen and optimized medium for each type of cells increased the migration speed significantly, the difference in migration direction remained. In EBM (the original medium used for HMEC-1s), HMEC-1s migrated toward the cathode as in DMEM, the standard medium we use for EF stimulation for all types of cells. However, the migration speed was increased significantly to ≈18 μm/h. BPAF cells in Eagle’s minimum essential medium with 15% FBS migrated toward the anode. Fibronectin and collagen coating significantly increased the migration rate of HUVECs and BPAF cells, respectively (≈14 μm/h). This confirmed the directional difference in microvascular and macrovascular cells.

To exclude possible chemotaxis effects in the chamber toward an electrically induced molecular gradient, we did control experiments with fluid flow perpendicular to the EF lines. A cross-flow of medium (0.6 mL/min) perpendicular to the EF through the chamber did not have significant effects on migration directedness of HMEC-1s. Cells still migrated toward the cathode in DC EF at 300 mV/mm (directedness 0.35±0.07; n=58). Thus, the applied EFs rather than any secondarily induced chemical gradient directed cell migration.

**Directional Migration of Vascular Cells Cultured in EFs Was Voltage Dependent**

We quantified the directional migration of the 4 types of vascular cells at different field strengths. The directedness of HMEC-1s migrating to the cathode and of BPAF cells, MASMCs, and HUVECs migrating to the anode all peaked at 150 to 200 mV/mm (Figure 3a). Additional increases of EF strength >200 mV/mm did not increase directedness, but instead directed migration of cells decreased (Figure 3a). Figure 3a indicates that vascular cells respond to EFs with a threshold ≈150 mV/mm.

**Small EFs Stimulated Migration of Vascular Cells**

Exposure to EFs significantly increased the translocation speed of cells; HMEC-1s increased the most (Figure 3b). At 200 mV/mm, HMEC-1s migrated fastest at ≈11 μm/h. This was ≈2× the rate of HUVECs (5.6 μm/h), 3× that of MASMCs (3.2 μm/h), and 20% higher than the rate of fibroblasts (9.2 μm/h; Figure 3b, 3d, and 3e). The increase in mean translocation rates for HMEC-1s and HUVEC showed a clear voltage dependency (Figure 3b).

**Small EFs Induced Alignment of Vascular Cells**

When cultured in EFs, HMEC-1s, BPAF cells, MASMCs, and HUVECs aligned with their long axis perpendicular to the EF vector (Figure 4b, 4d, 4f, and 4h). In control cultures (no EF), the long axis of cells aligned randomly (Figure 4a, 4c, 4e, and 4g). We quantified cell alignment using the Oi: Oi=cos 2α (see Methods and Figure 4i). The orientation response of all 4 types of vascular cells was time dependent in EFs, with a linear increase of Oi in EFs of 200 mV/mm (Figure 1a, available online at http://atvb.ahajournals.org). Orientation of each cell type was evident after 4 to 5 hours in the EF. BPAF cells, MASMCs,
and HUVECs showed a more robust response at 8 hours and 24 hours than HMEC-1s (Figure 1a).

The orientation response of the vascular cells also showed voltage dependence. BPAF, MASMC, and HUVEC cells had similar robust voltage dependency, whereas HMEC-1s showed a less dramatic increase in Oi, with time in an EF of 200 mV/mm (Figure 1b). However, for all the 4 types of vascular cells, the orientation response had a threshold between 50 and 150 mV/mm.

Small EFs Induced Elongation of Vascular Cells
Whereas the 4 types of vascular cells migrated and aligned in an EF, they also elongated in response to the EF (Figure 4b, 4d, 4f, and 4h). In contrast, cells cultured without applied EFs retained a typical nonpolarized morphology (Figure 4a, 4c, 4e, and 4g). We quantified the elongation of cells using the long:short axis ratio (see Methods). A perfectly round cell has a long:short axis ratio of 1. As cells elongate, the ratio increases. The elongation response to the EF happened much later than the directional migration and orientation of the long axis of the cell (Figure 1). Cultured in an EF of 200 mV/mm, cells did not show significant elongation after 5 hours; however, 24 hours after onset of continuous EF, HMEC-1s, BPAF cells, MASMCs, and HUVECs showed a significant elongation response when compared with control cells (0 mV for 24 hours; P<0.01 to 0.0001; Figure II, available online at http://atvb.ahajournals.org).

Discussion
DC EFs induce directional migration and a significant increase in cell migration rates of HMEC-1s and HUVECs, and of fibroblasts from bovine aorta and MASMCs. Intriguingly, HMEC-1s migrated in the opposite direction from endothelial cells, fibroblasts, and SMCs derived from large vessels. Each of the vascular cells tested showed EF-stimulated perpendicularly axis and cell elongation. Migration, orientation, and elongation of vascular cells are important cellular behaviors underlying angiogenesis and vascular remodeling. Our observation that applied EFs induced distinct heterogeneous responses in different vascular cells may be important in understanding the potential electrical control of angiogenesis by endogenous or exogenously applied electrical stimulation.

Blood flow to ischemic tissues can be re-established by 2 main mechanisms: angiogenesis and collateral circulation. Angiogenesis involves mainly endothelial sprouting from capillaries and venules. There is morphological and functional heterogeneity between endothelial cells from different parts of the body, from different sizes of vessels, in different organs, and in different regions of the same organ. One example is the different responsiveness of microvascular versus macrovascular endothelial cells to interleukin 8 (IL-8), a potent angiogenic agent. IL-8 binds chemokine receptors CXCR 1 to CXCR 3 to induce angiogenic response. HMEC-1s express more CXCR 1 to CXCR 3 and are more responsive to IL-8 than HUVECs, showing enhanced chemotactic and migratory response. Here we showed that contrasting differences also exist in the responses of HMEC-1s and HUVECs to a applied physiological EF. These include differences in migration direction, enhancement of migration speed, and the extent of cell reorientation and elongation. Each of these cell behaviors is potentially important for angiogenesis, therefore, clinical application of electrical stimulation should take account of this heterogeneity.

Different types of cells respond to EFs by migration toward different poles. Some cells migrate cathodally, for instance, neural crest cells, corneal epithelial cells, epidermal keratinocytes, pigmented retinal epithelium, embryonic fibroblasts, osteoblasts, and bovine aortic endothelial cells. Other cells migrate anodally, such as corneal endothelial cells, fibroblasts, osteoclasts, and peritoneal macrophages. Intriguingly, lens epithelial cells migrate either cathodally or anodally, depending on EF strength. How cells sense and transduce electric signals remains largely unknown. Because both HMEC-1s and HUVECs are endothelial cells and migrate in opposing directions at the same EF strength, they offer comparative models to dissect out the mechanisms that cells use to move directionally in a physiological EF.

The applied EFs of 150 to 400 mV/mm showed significant effects in directing vascular cell migration, orientation, and elongation. How does this relate to the EFs these cells will
experience in vivo? In pig heart, injured and ischemic tissues polarize electrically, and this can produce a DC EF of ≈5.8 mV/mm across an 8-mm zone at the boundary with undamaged tissue. It is unlikely that such a small EF will have any significant effects on angiogenesis in this particular situation. However, stronger endogenous EFs have been detected in other situations in which angiogenesis occurs in vivo. A steady DC EF of 100 to 200 mV/mm in murine skin arises as soon as the wound occurs, and this persists until re-epithelialization is complete. Malignancy of breast cancer has such an electric property change that noninvasive electropotential difference on skin surface can be detected. Whether those endogenous EFs have any effect on angiogenesis in vivo is yet to be determined.

Full maturation of new vessels into fully formed collateral circulation requires a vessel wall that contains SMCs and fibroblasts. These cell types have distinct roles and functional interrelations in regulating a variety of physiological functions, including vascular permeability and tone. We show here that EFs also have significant effects on basic cell behaviors such as directional migration, alignment, and elongation in these cell types.

In conclusion, small DC EFs of a size equivalent to those that arise immediately at a wound induced significant directional migration, orientation, and elongation responses of vascular endothelial cells, fibroblast cells, and SMCs. Distinct heterogeneity in the responses existed among the 4 types of vascular cells tested. This may have potential physiological and clinical implications in areas where electrical stimulation is used to promote angiogenesis or vasculature remodeling.

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Methods:

Electric Field Stimulation

Cells were seeded in the trough at a density of ~20 × 10^4 cells/ml and 0.5 ml/trough. A roof of number 1 cover glass (Chance Propper, Warley, England) was added and sealed with silicone grease 12-20 h after seeding. The final dimensions of this very shallow chamber, through which current was passed, were 22 mm × 10 mm × 0.2 mm. Agar-salt bridges were used to connect silver/silver chloride electrodes in beakers of physiological saline solution, to reservoirs of culture medium at the ends of the chambers. Field strengths were measured directly. One hour before EF stimulation, fresh complete DMEM was used to replace all media including EBM used for HMEC-1 culture. This kept identical medium conditions for all four types of cells tested.
Figure I. Time (a) and voltage (b) dependence of the orientation responses of vascular cells in electric fields. EF of 200mV/mm induced orientation of cells within 4 hours. By 8 hours and 24 hours, although microvascular cells aligned strongly perpendicular to the EF, each of the other cell types showed a more robust response (a). The insert shows the response curves of the cells in different time-points at 200 mV / mm EF strength. *p<0.05 and **p<0.01; # P<0.05-0.000 when compared with corresponding controls(0 hour) after 4, 8, and 24 hours EF exposure, respectively. n= 65-598 from at least two independent experiments. b. Voltage dependence of vascular cells at individual field strengths of 0-400 mV over 5 hours. The number of cells measured for each point are 57~103 , 56~115, 103~116 and 82~114 for HMEC-1, BPAF, MASMC and HUVEC, respectively. *p<0.01-0.0001 when compared with corresponding controls with no EF (0 mV).
Figure II. EF induced elongation of vascular cells. Elongation was quantified as the long/short axis ratio of individual cells and did not show significant change until 24 hours of EF exposure. These cell types showed an increased elongation when compared with corresponding control cells (24hr: 0mV) (*P<0.01- 0.0001). n= 47-343 from at least two independent experiments. Values are means ± S.E.M.