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Interleukin-1 β -mediated inhibition of the processes of angiogenesis in cardiac microvascular endothelial cells

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Abstract

Angiogenesis, the formation of new capillaries from preexisting vessels, plays an essential role in revascularization of the myocardium following myocardial infarction (MI). Interleukin-1 β (IL-1 β), a proinflammatory cytokine increased in the heart following MI, is shown to be essential for angiogenesis in the invasiveness of tumor cells, the progression of arthritic conditions and endometriosis, and the promotion of wound healing. Here we studied the steps of angiogenesis in response to IL-1 β in cardiac microvascular endothelial cells (CMECs) and aortic tissue. Cell cycle progression analysis using flow cytometry indicated a G₀/G₁ phase cell cycle arrest in IL-1 β -stimulated cells. IL-1 β significantly reduced levels of fibrillar actin in the cytoskeleton, a pre-requisite for tube formation, as indicated by phalloidin-FITC staining. Wound healing assays demonstrated IL-1 β prevents cell-to-cell contact formation. On the other hand, vascular endothelial growth factor-D (VEGF-D) initiated restoration of the cell monolayer. IL-1 β significantly inhibited in vitro tube formation as analyzed by three-dimensional collagen matrix assay. Aortic ring assay demonstrated that IL-1 β inhibits basal and VEGF-D-stimulated microvessel sprouting from aortic rings. The data presented here are novel and of significant interest, providing evidence that IL-1 β impedes the process of angiogenesis in myocardial endothelial cells.

Keywords

Interleukin-1 β ; VEGF-D; Angiogenesis

Introduction

Angiogenesis, the formation of new capillaries from preexisting vessels, plays an essential role in development and pathological conditions such as wound healing, tumor growth and metastasis, and revascularization of the myocardium following myocardial infarction (MI) (Isner and Losordo, 1999). Formation of new blood vessels is critical for supplying the healing infarcted myocardium with oxygen and nutrients to sustain metabolism. The process of angiogenesis consists of several steps: stimulation of endothelial cells by growth factors, degradation of the extracellular matrix (ECM) by proteolytic enzymes at the basement membrane of endothelial cells, endothelial cell migration, proliferation, and invasion of the ECM, and finally the formation of new capillary tubes (Carmeliet, 2000). Microvascular

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endothelial cells of the smallest vessels or capillaries are integral players in the processes of angiogenesis.

The initiation of angiogenesis, the angiogenic switch, is dependent on a dynamic balance between proangiogenic and antiangiogenic factors in the endothelial cell environment (Hanahan and Folkman, 1996). Interleukin-1 β (IL-1 β), a proinflammatory cytokine, is shown to be essential for angiogenesis in the invasiveness of tumor cells, the progression of arthritic conditions and endometriosis, and the promotion of wound healing (Arend and Dayer, 1995; Geiger et al., 1993; Lebovic et al., 2000; Voronov et al., 2003). In contrast, our recent studies indicate IL-1 β inhibits expression of vascular endothelial growth factor-D (VEGF-D), shown to promote myocardial angiogenesis and believed to be the most potent angiogenic growth factor in the VEGF family (Mountain et al., 2007b; Rissanen et al., 2003; Rutanen et al., 2004). Inflammatory cytokines, such as IL-1 β and tumor necrosis factor- α (TNF- α), are increased in the heart during chronic heart failure (Ukimura et al., 2003) and following MI (Ono et al., 1998; Yue et al., 1998). IL-1 β is considered to play an important role in myocardial remodeling. Neutralization of IL-1 β in the acute phase of MI suppressed procollagen α_1 (III) gene expression and increased left ventricular dilation (Hwang et al., 2001). In contrast, overexpression of IL-1 receptor antagonist is shown to protect myocardium from ischemia/reperfusion injury by attenuating the inflammatory response associated with decreased apoptosis (Suzuki et al., 2001). Increased levels of IL-1 β are correlated with increased degree of interstitial fibrosis (Ono et al., 1998). In vitro, IL-1 β induces cardiac myocyte hypertrophy (Palmer et al., 1995; Petersen and Burleigh, 2003; Thaik et al., 1995) but exerts a potent anti-proliferative effect in cardiac fibroblasts (Palmer et al., 1995).

The present study was undertaken to examine IL-1 β -stimulated angiogenic activity in cardiac microvascular endothelial cells (CMECs), a major cell type in the processes of cardiovascular angiogenesis. Here we identified that IL-1 β delays cells cycle progression, prevents cell-to-cell contact formation and wound healing, decreases levels of fibrillar actin in the cell cytoskeleton, inhibits in vitro tube formation, and decreases vessel sprouting from an aortic ring.

Materials and Methods

Isolation and culture of CMECs

Adult rat CMECs were isolated as described (Xie et al., 2001), with minor modifications. Briefly, hearts from adult male Sprague-Dawley rats (200–225g) were removed under sterile conditions and perfused with DMEM supplemented with 0.1% penicillin-streptomycin (PS). After removing atria, visible connective tissue, valvular tissue, and the right ventricle, the left ventricle was immersed in 70% ethanol for 10sec to devitalize epicardial mesothelial and endocardial endothelial cells. After peeling away the outer one-third of the ventricular wall, the remaining tissue was washed in Hanks' balanced salt solution (HBSS). The tissue was finely minced and digested in 15ml HBSS containing 30mg collagenase at 37°C with gentle shaking for 20min. After a second digestion under the same conditions with the addition of 3mg trypsin, the solution was passed through an 80 μ m nylon mesh to remove undigested tissue. The dissociated cells were pelleted at 1050 rpm for 5min, washed in HBSS, resuspended in DMEM supplemented with 0.2% PS and 20% heat-inactivated FBS, and plated on laminin (10 μ g/ml) coated dishes or coverslips. The culture medium was replaced after 1h to remove nonadherent cells. Using Griffonia Simplicifolia Lectin-1 cytochemical staining we found that the CMECs culture is $\geq$ 95% pure. The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No.85-23, revised 1996). The animal protocol was approved by the University Committee on Animal Care. IL-1 β concentrations used in this study were selected based on previously published in vitro data in this and other cell types, including that indicating IL-1 β effects the

expression of VEGF-D in a concentration dependent manner (Mountain et al., 2007b; Maruyama et al., 1999; Singh et al., 1996). Of note, plasma levels of IL-1 β studied in 24 MI patients over the course of 96h showed peak levels at 22.2 $\pm$ 8.6 pg/ml compared to <10 pg/ml in noninfarcted patients (Pudil et al., 1999). In rat heart, IL-1 β is suggested to be synthesized by macrophages, endothelial cells, and vascular smooth muscle cells (Ono et al., 1998). It is likely that these cells are exposed to a higher concentration of IL-1 β than what is seen in the plasma of patients with myocardial infarction. VEGF-D (50ng/ml) in this study was used merely as a positive control treatment group, therefore concentration response curves were not performed.

Cell Cycle Progression Assay

CMECs were seeded in 60mm culture dishes at a density of 5×10^4 cells/dish and incubated in DMEM supplemented with 1% heat-inactivated FBS for 24 hours at 37°C to slow down cell cycle progression in order for IL-1 β effects to be measured. Quiescence could not be induced prior to IL-1 β exposure because serum starvation would itself cause a cell cycle delay and interfere with IL-1 β analysis. The cells were then treated with IL-1 β (4ng/ml; R&D Systems, Inc., Minneapolis, MN), in serum-free DMEM for 24h. The cells were then trypsinized and fixed with 70% ethanol in PBS at 4°C overnight. Cells were then centrifuged and incubated in 1ml staining solution (50 μ g/ml propidium iodide, 100 μ g/ml RNaseA, and 0.1% Triton X-100 in PBS) for 30 minutes at 37°C in the dark. Cells were analyzed by flow cytometry with excitation at 488 nm and emission measured at 560 to 640 nm. A minimum of 5×10^4 cells were analyzed from each sample, and the percentage of cells in G₀/G₁ phase was determined using CellQuest software (Becton Dickinson; San Jose, CA). Control cells (C) were treated with equal volumes of IL-1 β delivery vehicle (0.1% BSA/PBS), and cells treated with DMEM containing 20% FBS served as a positive control.

Actin Polymerization Assay

CMECs were grown to 70% confluency on coverslips, made quiescent by serum starvation for 24h, and treated with IL-1 β (4ng/mL; 24h) for 24h. Cells were washed twice with phosphate-buffered saline (PBS), fixed in 3.7% formaldehyde solution in PBS for 10min at room temperature, and permeabilized with 0.1% Triton X-100. Nonspecific binding was blocked by incubating slides for 20min at room temperature in blocking solution (1% BSA in PBS). Cells were stained with Phalloidin-FITC (1U/slide in blocking solution; Sigma-Aldrich, St. Louis, MO) for 20min at room temperature in the dark. After washing, the slides were mounted with SlowFade mountant (Invitrogen Corp., Carlsbad, CA) and visualized under fluorescence microscopy using a rhodamine filter. Images were acquired using a Nikon TE-2000 microscope with a Retiga-1300 color cooled camera. The actin structure was analyzed using Bioquant Image Analysis software (Bioquant Image Analysis Corp., Nashville, TN).

Wound Healing Assay

Movement of cells through a wound introduced in a cell monolayer was determined as described (Hochman et al., 2006). Briefly, CMECs were grown as a confluent monolayer. After cells were made quiescent in serum free medium for 24h, a wound was created in the center of the cell monolayer by gentle removal of attached cells using a sterile plastic pipette tip. Cell debris was removed by a PBS wash and images of the wound were acquired using a Nikon TE-2000 microscope with a Retiga-1300 color cooled camera. The cells were then incubated in serum-free DMEM containing IL-1 β (4ng/ml) and/or VEGF-D (50ng/ml) for 24h. Images were again acquired using the setup as described above. The wound area was measured using Bioquant Image Analysis software. The ability of cells to migrate into the wound area was assessed by comparing micrographs at time zero and 24h along the wounded area. The

percentage of non-recovered wound area was calculated by dividing the wound area after 24h by the initial wound area at time zero, multiplied by 100.

In Vitro Tube Formation Assay

Three-dimensional cultures of CMECs were established as described (Sierra-Honigmann et al., 1998) with minor modifications. Gel matrices were prepared on ice in DMEM supplemented with 0.1% PS by the addition of rat tail Type I collagen (1.75 mg/ml) and fibronectin (90µg/ml). CMECs were added to a final concentration of 1×10^6 cells/ml. Twenty-four well plates were immediately coated with 250µl of the cell-collagen mixture and placed in a humidified CO₂ incubator at 37°C for 15min to allow them to solidify. DMEM (500µl) supplemented with 1% heat-inactivated FBS with or without IL-1β (4ng/ml) was then added to each well. Quiescence with serum free medium was not induced due to the lengthy duration of experiments. Medium and treatment was replaced after 24h. CMECs were allowed to form tubular structures for 48h in culture. Images were acquired using a Nikon TE-2000 microscope with a Retiga-1300 color cooled camera.

Aortic Ring Sprouting Assay

Matrigel (growth factor reduced BD Matrigel Matrix, BD Biosciences, San Jose, CA) was thawed at 4°C overnight. A cooled pipette was used to mix the Matrigel to homogeneity. Aortic ring assays were performed as described (Nicosia and Ottinetti, 1990) with minor modifications. Aortas were removed from adult male Sprague-Dawley rats (200–225g) and immediately placed in ice cold DMEM supplemented with 0.1% PS. Aortas were cleaned of surrounding connective tissue and sliced into 1mm thick rings under a dissecting microscope. Ninety-six well plates were coated with 60µl of Matrigel and placed at 37°C for 10min to gel. Aortic rings were placed in wells, sealed in place with a 20µl Matrigel overlay, and again placed at 37°C for 10min to gel. Serum-free DMEM (100µl) containing IL-1β (4ng/ml), VEGF-D (50ng/ml), or both was added. Serum free medium was used because growth factors necessary to sustain tissue for the lengthy duration of experiments were provided via the Matrigel matrix. Medium alone and medium supplemented with 20% heat-inactivated FBS served as controls. Aortic rings were incubated at 37°C in 5% CO₂ for 10 days to allow microvessel sprouting. Medium and treatment was replaced every 48h. Images were acquired using a Nikon TE-2000 microscope with a Retiga-1300 color cooled camera. Arterial rings were measured using Bioquant Image Analysis software. Sprouting was analyzed by calculating the percent of arterial ring circumference (exterior and interior) occupied by microvessel sprouts.

Statistical analysis

All data are reported as mean±SEM. Statistical analyses were performed using Student's *t*-test or one-way ANOVA and a post hoc Tukey's test. Probability (*P*) values of <0.05 were considered to be significant.

Results

IL-1β arrests CMECs at G₀/G₁ phase of the cell cycle

Endothelial cell proliferation is necessary for the formation of new vasculature. Analysis of cell cycle progression using flow cytometry indicated the presence of ~60% of control cells in G₀/G₁ phase (Fig 1A). IL-1β consistently resulted in ~10% increase in the number of cells in G₀/G₁ phase of the cell cycle (Fig 1B), indicating an arrest in cell cycle progression. Cells cultured in DMEM supplemented with 20% FBS served as a positive control, consistently decreasing the number of cells in G₀/G₁ phase (Fig 1C).

IL-1 β inhibits actin polymerization in CMECs

Alterations in the actin cytoskeleton may alter the protrusive force required for the outgrowth and migration of endothelial cells (Ridley et al., 2003). Analysis of the actin cytoskeleton using phalloidin-FITC staining indicated a sharp reduction in fibrillar actin after 24h of IL-1 β stimulation (Fig. 2A). Quantitative analysis of four separate fields in eight independent cell preparations revealed a significant decrease in actin polymerization (pixels/cell; control, 1051 $\pm$ 128; IL-1 β , 332 $\pm$ 76*; * P <0.001 vs. control; n=8; Fig. 2B).

IL-1 β prevents wound healing in vitro

The significant absence of fibrillar actin in IL-1 β -stimulated cells suggests a defect in cytoskeleton organization and migration. However, it has been shown that IL-1 β is essential for invasiveness of melanoma cells and mammary adenocarcinoma cells (Voronov et al., 2003). IL-1 β has also been shown to drastically increase migration of vascular smooth muscle cells in vitro (Wen et al., 2002). To examine the functional effects of IL-1 β , we used an in vitro wound (scratch) assay. This assay is commonly used for testing the effects of pro- and anti-migratory agents on cultured cells (Etienne-Manneville and Hall, 2001; Raftopoulou et al., 2004). CMECs were grown to confluence and a wound was introduced into the cell monolayer. IL-1 β enhanced the movement of individual CMECs into the wounded area after 24h (Fig. 3A). However, there was almost no cell-to-cell contact among the migrated cells and thin spindle projections from cell bodies can be observed. For measuring wound area the border of the wound was considered to be the points at which the cell monolayer was disrupted. IL-1 β significantly prevented healing of the wound (percent non-recovered wound area; control, 69 $\pm$ 6; IL-1 β , 93 $\pm$ 3*; * P <0.05 vs. control; n=7; Fig. 3B). VEGF-D enhanced recovery of the wounded area, significantly restoring the cell monolayer after 24h, while IL-1 β significantly prevented this stimulatory effect (percent non-recovered wound area; VEGF-D, 34 $\pm$ 8*; VEGF-D1 β , 75 $\pm$ 10[#]; * P <0.05 vs. control (C); [#] P <0.05 vs. VEGF-D; n=4–7; Fig. 3A and B).

IL-1 β prevents the formation of capillary-like tubes in three-dimensional culture

The processes of angiogenesis ultimately must culminate in individual endothelial cells coming together to form a cohesive tubular structure. The lack of cell-to-cell contact, the disassembly of cytoskeletal actin, and cell cycle arrest indicate a phenotype opposite to functional angiogenesis. In order to examine the effect of IL-1 β on tube formation in vitro, cells were embedded in a three-dimensional collagen matrix. This analysis demonstrated that CMECs in the presence of IL-1 β form fewer capillary-like structures than control cells (n=6). Figure 4 shows capillary-like structures obtained from two independent experiments.

IL-1 β inhibits angiogenic sprouting of aortic tissue

CMECs are considered major players in the processes of angiogenesis. Other cell types are also suggested to participate in these processes. For example, smooth muscle cells are recruited to “muscularize” and stabilize newly formed vessels (Carmeliet, 2000). Fibroblasts, along with other cell types, play an important role in degradation and laying down new components of the extracellular matrix (Eghbali et al., 1989; Eghbali et al., 1988). Here we used a rat aortic ring assay to examine the effect of IL-1 β on angiogenesis where multiple cell types are present (Diglio et al., 1989). Using the criteria described in “Materials and Methods,” we show that IL-1 β inhibited basal and VEGF-D-stimulated microvessel sprouting from the cultured aortic rings (percent arterial ring circumference occupied; control, 32 $\pm$ 3; IL-1 β , 12 $\pm$ 3*; VEGF-D, 48 $\pm$ 4*; VEGF-D1 β , 19 $\pm$ 4[#]; * P <0.05 vs. control (C); [#] P <0.05 vs. VEGF-D; n=3–4; Fig. 5A and B).

Discussion

Angiogenesis, the formation of new blood vessels from pre-existing vasculature, is essential in the repair of the heart post MI. Angiogenesis involves a series of essential processes for functional blood vessel formation. IL-1 β , a proinflammatory cytokine increased in the heart post-MI, has been shown to play a pro-angiogenic role in a variety of conditions (Arend and Dayer, 1995; Geiger et al., 1993; Lebovic et al., 2000; Ono et al., 1998; Voronov et al., 2003; Yue et al., 1998). This study was undertaken to examine the role of IL-1 β in the regulation of the processes of angiogenesis in CMECs, a cell type of central interest in cardiovascular angiogenesis. The major findings of this study are 1) IL-1 β inhibits cell cycle progression; 2) IL-1 β inhibits cytoskeletal actin polymerization; 3) IL-1 β prevents cell-to-cell contact and wound healing; 4) IL-1 β prevents tube-formation in three-dimensional culture; 5) IL-1 β inhibits microvessel sprouting from aortic tissue. Collectively, these studies suggest that IL-1 β may inhibit angiogenesis in the heart post-MI.

Endothelial cell proliferation is necessary for the formation of new vasculature. Cell proliferation can be negatively affected by two mechanisms: enhanced apoptosis and cell cycle progression arrest. Measurement of apoptosis using TUNEL-staining and Annexin-V-staining assays indicated that IL-1 β -stimulation (24h) does not induce apoptosis in CMECs (data not shown). Flow cytometric analyses of cells demonstrated that IL-1 β arrests cells in G₀/G₁ phase of cell cycle. These data suggest that IL-1 β may negatively affect new vessel formation by causing cell cycle arrest at G₀/G₁, possibly resulting in a decrease in DNA replication and cellular mitosis.

Cell migration plays a central role in a wide variety of biological conditions. It is essential in embryogenesis and remains prominent in the adult for the inflammatory response, wound healing, tumor metastasis, etc (Lauffenburger and Horwitz, 1996). The initial migratory response of a cell is to polarize and form cellular protrusions, such as filopodia and lamellipodia, which push forward the leading edge so that the cell invades the surrounding tissue. The formation of these protrusions requires the polymerization of actin, and their stability is dependent upon adherence to the ECM or adjacent cells via transmembrane receptors linked to the actin cytoskeleton (Ridley et al., 2003). Furthermore, the actin cytoskeleton is anchored to focal adhesions which function as cellular contact points for communication with the ECM to allow changes in response to integrin- and growth-factor-mediated signals (Brakebusch and Fassler, 2003). Therefore, it is apparent that disruption in the organization of the actin cytoskeleton could have widespread deleterious effects on normal angiogenic processes. In this study we show that IL-1 β severely disrupts fibrillar actin organization in CMECs, indicating the possibility for interference in cell migration and cell signaling.

In order for a cell to migrate, it must free itself from the ECM and neighboring cells. Matrix metalloproteinases (MMPs) are a family of proteolytic enzymes that promote ECM degradation and remodeling. In liposarcoma cells, IL-1 β -stimulated activation of MMP-2 and -9 is shown to increase cell invasiveness (Pazzaglia et al., 2004). We have previously shown that IL-1 β increases the expression and activity of MMP-2, not MMP-9, in CMECs (Mountain et al., 2007a). Here we show that IL-1 β facilitates non-directed individual cell movement within a wounded area, however cell-to-cell contact and wound healing is prevented. This increase in cell motility could be due to the increased activity of MMP-2, freeing the cells from their anchoring matrix. In the wounded area thin spindle projections from the cell body can be observed in IL-1 β -stimulated CMECs. These projections are most likely abnormally formed filopodia and lamellipodia due to the inhibition of actin polymerization. This abnormal formation of filopodia and lamellipodia may interfere with cell-to-cell and/or cell-to-ECM contact formation. For restoration of a cell monolayer or functional tube formation, cell-to-cell

contact must be re-established. The absence of cell-to-cell contact formation in IL-1 β -stimulated CMECs indicates a dysfunctional phenotype for angiogenesis. Of note, IL-1 β has been shown to decrease synthesis of the major fibrillar procollagens $\alpha_1(I)$, $\alpha_2(I)$, and $\alpha_1(III)$ in cardiac fibroblasts (Siwik et al., 2000). Collagen is the major determinant of myocardial structural integrity (Weber et al., 1994), and fibroblasts are the major sites for its synthesis (Eghbali et al., 1989; Eghbali et al., 1988). While ECM degradation is essential for cellular migration, new basement membrane components must be deposited to support newly formed blood vessel maturation. Lack of cell-to-cell contact formation indicates that IL-1 β may inhibit the formation of functional vessels. In fact, three dimensional in vitro tube formation assays demonstrated the absence of short branching tubular structures in IL-1 β -stimulated cultures, suggesting an anti-angiogenic role for IL-1 β in CMECs. These findings were further supported by ex vivo aortic ring assays, where IL-1 β significantly inhibited basal microvessel sprouting from aortic ring cultures.

VEGFs, a multigene family of proteins, have been found to be potent endothelial cell mitogens and key regulators of angiogenesis (Battegay, 1995; Ferrara, 1996). Upon adenoviral transfection of rabbit hindlimb skeletal muscle, VEGF-D was found to be the strongest inducer of angiogenic activity of all the VEGF isoforms (Rissanen et al., 2003). VEGF-D has been shown to induce proliferation of bovine aortic endothelial cells (Achen et al., 1998) and proliferation and migration of HUVECs (Byzova et al., 2002; Marconcini et al., 1999). Intramyocardial injection of adenoviruses encoding VEGF-D has been shown to promote transmural angiogenesis with marked increase in myocardial perfusion (Rutanen et al., 2004). Since IL-1 β inhibits the expression of VEGF-D in CMECs (Mountain et al., 2007b), we were expecting exogenous VEGF-D to restore wound healing and microvessel sprouting in the presence of IL-1 β . Instead, IL-1 β inhibited VEGF-D-stimulated increases in microvessel sprouting and wound healing. These data suggest involvement of additional factor/s other than VEGF-D in IL-1 β -stimulated inhibition of angiogenic processes.

Conclusion

The data presented here are novel and of significant interest in that IL-1 β is considered an important pro-angiogenic cytokine in a variety of pathologies. Here we provide evidence that IL-1 β inhibits a number of angiogenic processes in cardiac microvascular endothelial cells and inhibits tube formation. In vivo studies of myocardial injury could further elucidate the specific roles for IL-1 β in cardiovascular angiogenesis and is area of future investigation in our laboratory. A clear understanding of the regulation of angiogenic processes by cytokines and growth factors in the immediate environment is essential for the development of pharmacological strategies targeting functional remodeling and angiogenesis of the myocardium post-MI.

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Figure 1. IL-1 β arrests CMECs at G₀/G₁ phase of the cell cycle

CMECs, seeded at a density of 5×10^4 cells/dish, were stimulated with IL-1 β (4ng/ml) for 24h. DMEM supplemented with 20% FBS served as a positive control. The cells were trypsinized, stained with propidium iodide, and cell cycle progression was analyzed by flow cytometry. Shown are DNA profiles of control cells (A), IL-1 β -treated cells (B), and FBS-treated cells (C), representative of four independent experiments. Percentages are mean $\pm$ SEM, n=4.

Figure 2. IL-1 β decreases actin polymerization in CMECs

Semi-confluent cultures of CMECs were treated with IL-1 β (4ng/ml) for 24h, stained with Phalloidin-FITC (1U), and visualized under fluorescence microscopy. **Panel A** demonstrates representative images from eight independent experiments. Fluorescence was quantitatively analyzed as pixels per cell. **Panel B** represents the mean data $\pm$ SEM. * $P < 0.001$ vs. control (C); $n = 8$.

Figure 3. IL-1 β inhibits CMEC wound healing

Confluent CMEC monolayers were wounded using a sterile pipette tip and treated with IL-1 β (4ng/ml) and/or VEGF-D (50ng/ml). The ability of cells to migrate into the wound was assessed following 24h of treatment. **Panel A** demonstrates the wound area at 0h and 24h after treatment. Wound healing was assessed by calculating percent of non-recovered wound area. **Panel B** represents the mean data $\pm$ SEM. * $P < 0.05$ vs. control (C); # $P < 0.05$ vs. VEGF-D; n=4–7.

Figure 4. IL-1 β inhibits three-dimensional tube formation

CMECs were seeded at a concentration of 1×10^6 cells/ml in three-dimensional gel matrices of collagen and fibronectin. Cultures were treated with IL-1 β (4ng/ml) and allowed to form tubular structures for 48h. Images shown are from two replicates and are representative of six independent experiments. Arrows indicate tubular structures.

Figure 5. IL-1 β inhibits microvessel sprouting from aortic rings

Aortas were removed from adult male rats and immediately sliced into 1mm thick rings. Rings were embedded in Matrigel, treated with IL-1 β (4ng/ml) and/or VEGF-D (50ng/ml), and cultured for 10 days. DMEM supplemented with 20% FBS served as a positive control. **Panel A** demonstrates representative images from 4 independent experiments. Sprouting was assessed by calculating percent of arterial ring circumference occupied by sprouts. **Panel B** represents the mean data $\pm$ SEM. * P <0.05 vs. control (C); # P <0.05 vs. VEGF-D; n=3–4.