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Interleukin-1β-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-1β (IL-1β), 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-1β in cardiac microvascular endothelial cells (CMECs) and aortic tissue. Cell cycle progression analysis using flow cytometry indicated a G_0/G_1 phase cell cycle arrest in IL-1β-stimulated cells. IL-1β 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-1β prevents cell-to-cell contact formation. On the other hand, vascular endothelial growth factor-D (VEGF-D) initiated restoration of the cell monolayer. IL-1β significantly inhibited in vitro tube formation as analyzed by three-dimensional collagen matrix assay. Aortic ring assay demonstrated that IL-1β 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-1β impedes the process of angiogenesis in myocardial endothelial cells.

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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 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 pro-angiogenic and anti-angiogenic factors in the endothelial cell environment (Hanahan and Folkman, 1996). Interleukin-1β (IL-1β), 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-1β 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., in press; Rissanen et al., 2003; Rutanen et al., 2004). Inflammatory cytokines, such as IL-1β 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-1β is considered to play an important role in myocardial remodeling. Neutralization of IL-1β 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-1β are correlated with increased degree of interstitial fibrosis (Ono et al., 1998). In vitro, IL-1β 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-1β-stimulated angiogenic activity in cardiac microvascular endothelial cells (CMECs), a major cell type in the processes of cardiovascular angiogenesis. Here we identified that IL-1β delays cells cycle progression, prevents cell-

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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 (200–225 g) 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 10 s 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 15 ml HBSS containing 30 mg collagenase at 37 °C with gentle shaking for 20 min. After a second digestion under the same conditions with the addition of 3 mg trypsin, the solution was passed through an 80 μm nylon mesh to remove undigested tissue. The dissociated cells were pelleted at 1050 rpm for 5 min, washed in HBSS, resuspended in DMEM supplemented with 0.2% PS and 20% heat-inactivated FBS, and plated on laminin (10 μg/ml) coated dishes or coverslips. The culture medium was replaced after 1 h to remove nonadherent cells. Using Griffonia Simplicifolia Lectin-1 cytochemical staining we found that the CMECs culture is ≥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-1β concentrations used in this study were selected based on previously published in vitro data in this and other cell types, including that indicating IL-1β effects the expression of VEGF-D in a concentration dependent manner (Mountain et al., in press; Maruyama et al., 1999; Singh et al., 1996). Of note, plasma levels of IL-1β studied in 24 MI patients over the course of 96 h showed peak levels at 22.2±8.6 pg/ml compared to <10 pg/ml in noninfarcted patients (Pudil et al., 1999). In rat heart, IL-1β 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-1β than what is seen in the plasma of patients with myocardial infarction. VEGF-D (50 ng/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 60 mm culture dishes at a density of 5×10^4 cells/dish and incubated in DMEM supplemented with 1% heat-inactivated FBS for 24 h at 37 °C to slow down cell cycle progression in order for IL-1β effects to be measured. Quiescence could not be induced prior to IL-1β exposure because serum starvation would itself cause a cell cycle delay and interfere with IL-1β analysis. The cells were then treated with IL-1β (4 ng/ml; R&D Systems, Inc., Minneapolis, MN), in serum-free DMEM for 24 h. The cells were then trypsinized and fixed with 70% ethanol in PBS at 4 °C overnight. Cells were then centrifuged and incubated in 1 ml staining solution (50 μg/ml propidium iodide, 100 μg/ml RNaseA, and 0.1% Triton X-100 in PBS) for 30 min 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 G_0/G_1 phase was determined using CellQuest software (Becton Dickinson; San Jose, CA). Control cells (C) were treated with equal volumes of IL-1β 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 24 h, and treated with IL-1β (4 ng/mL; 24 h) for 24 h. Cells were washed twice with phosphate-buffered saline (PBS), fixed in 3.7% formaldehyde solution in PBS for 10 min at room temperature, and permeabilized with 0.1% Triton X-100. Nonspecific binding was blocked by incubating slides for 20 min 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 20 min 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 24 h, 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-1β (4 ng/ml) and/or VEGF-D (50 ng/ml) for 24 h. 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 24 h along the wounded area. The percentage of non-recovered wound area was calculated by dividing the wound area after 24 h 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 (90 μg/ml). CMECs were added to a final concentration of 1×10^6 cells/ml. Twenty-four well plates were immediately coated with 250 μl of the cell-collagen mixture and placed in a humidified CO_2 incubator at 37 °C for 15 min to allow them to solidify. DMEM (500 μl) supplemented with 1% heat-inactivated FBS with or without IL-1β (4 ng/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 24 h. CMECs were allowed to form tubular structures for 48 h 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 (200–225 g) and immediately placed in ice cold DMEM supplemented with 0.1% PS. Aortas were cleaned of surrounding connective tissue and sliced into 1 mm thick rings under a dissecting microscope. Ninety-six well plates were coated with 60 μl of Matrigel and placed at