The American Journal of Surgery 192 (2006) 604–609
Presentation

Sustained low levels of fibroblast growth factor-1 promote persistent microvascular network formation

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Manuscript received May 22, 2006; revised manuscript August 2, 2006

Presented at the 30th Annual Surgical Symposium of the Association of VA Surgeons, Cincinnati, Ohio, May 7–9, 2006

Abstract

Background: Therapeutic neovascularization using high growth factor concentrations may lead to transient vessel formation and abnormal microvascular structure. The goal of this study was to quantify temporal and concentration effects of fibroblast growth factor-1 (FGF-1) on the persistence and morphology of microvascular networks.

Methods: Endothelial cells were incubated in suspension culture forming aggregates that were embedded in fibrin glue (FG) and stimulated with varying concentrations of FGF-1 with of heparin. Capillary networks formed were quantified for 21 days.

Results: High FGF-1 concentrations resulted in rapid and intense sprout formation, with excessive branching. At later times, these vessels regressed, with cellular debris in former vessel locations. At later times, the 1-ng/mL group surpassed the high concentration groups with continuous sprout growth and complete FG vascularization by 23 days.

Conclusion: Sustained low levels of FGF-1 maintained a persistent microvascular network response, whereas higher levels resulted in abnormal phenotype followed by vessel regression.

Keywords: Neovascularization; Angiogenesis; FGF-1; Endothelial cells; Capillary regression

The microcirculatory system provides oxygen, nutrients, and immune cells while removing metabolic waste from all tissues in our body. Neovascularization, the formation of new blood vessels, plays a crucial role in both normal and pathologic tissue function [1]. The ability to therapeutically control this phenomenon could be of enormous benefit to the treatment of a number of disease states.

Neovascularization occurs via 2 major mechanisms: angiogenesis and vasculogensis. Vasculogenesis is the in situ assembly of precursor cells including angioblasts and endothelial precursor cells into capillaries and larger vascular structures, whereas angiogenesis is the formation of new capillaries from preexisting vessels [2]. Angiogenesis and vasculogenesis were initially considered independent events; however, it is now recognized that neovascularization is a more complex process in which both mechanisms may contribute to vessel formation within a single microenvironment. Arteriogenesis is also a mechanism of clinical importance for ischemic disease. Although arteriogenesis is not neovasculzarization, it is the process by which preexisting vessels are remodeled into larger vessels for increased conductance.

Neovascularization and arteriogenesis are promoted by multiple growth factors and cytokines including members of the fibroblast growth factor (FGF) family. FGFs are mitogenic for a broad range of cell types including endothelial cells (ECs), smooth muscle cells (SMCs), and fibroblasts [3]. The mechanism in which proteins of the FGF family regulate EC growth and migration is crucial to the understanding of the angiogenesis phenomenon and the possible treatment for many cardiovascular diseases including myocardial and peripheral limb ischemia [4].

Supported by grants from the NIH (R01-HL 41272, F32-HL074594) and the Veterans Administration (HPG, EMB).

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0002-9610/06/$ – see front matter
doi:10.1016/j.amjsurg.2006.08.012

The FGF family is comprised of nine naturally occurring proteins with structurally related polypeptide sequences. Many of the FGF family proteins are considered oncogenes and are thus mitogenic for a wide variety of cell types [5]. The most studied FGF proteins are FGF-1 (acidic FGF) and FGF-2 (basic FGF). Both promote angiogenesis through interactions with high-affinity FGF tyrosine kinase receptors and low affinity high abundance heparan sulfate proteoglycans on cell surfaces [5].

The use of FGFs in neovascularization therapeutics has had disappointing results when translating success in animal models to clinical application. One possible explanation for the inconclusive results are the high growth factor concentrations administered [6,7]. The success of neovascularization therapies is dependent on sustained angiogenic response with normal vessel geometry and functional properties. Recent studies have shown that it is the local microenvironmental concentration and not the total dose that determines the resultant microvascular structure [8]. The goal of this study was to quantify the effect of FGF-1 concentration on formation of microvasculuar networks. Additionally, the effect of FGF-1 concentration on the persistence and morphologic characteristics of the induced microvascular network formation was studied.

Materials and Methods

Materials

Chemicals, biological reagents, and experimental supplies were obtained as follows: 0.05% trypsin/EDTA; Hank's balanced salt solution (HBSS); collagenase and fungisone from Gibco (Grand Island, NY); fibronectin, fibrinogen, and human thrombin from the American Red Cross (Rockville, MD); bBovine lung heparin from Upjohn (Kalamazoo, MI); methylcellulose and aprotinin from Sigma (St Louis, MO); woven nylon mesh rings (ID = 7.5 mm, OD = 13 mm) from Sefar America Inc (Kansas City, MO); Parafilm M from (American National Can, Greenwich, CT); 100-mm Petri dishes from Fisher Scientific (Pittsburgh, PA); 24 wells plates; and tissue culture flasks from Corning Costar Corp (Cambridge, MA). EC complete medium consisted of M199 (Gibco), 10% fetal bovine serum (Hyclone, Logan, UY), 100 U/mL penicillin, and 100 μ/mL streptomycin. Recombinant FGF-1 was produced and purified as described previously [9].

Animal care

All animal procedures complied with *The Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, Commission of Life Sciences, National Research Council, 1996) and *The Principles of Laboratory Animal Care* (National Institutes of Health publication no. 85-23, revised 1985).

Cell isolation

Canine jugular vein ECs (CJVECs) were harvested from adult, mongrel dogs' jugular veins. The dogs were anesthetized with thiopental sodium, intubated, and ventilated. Anesthesia was maintained with nitrous oxide and isofluorane. Bilateral neck incisions were made and the external jugular veins removed and processed as described previously [8,9]. Briefly, canine jugular veins were inverted and placed in HBSS and spun at 800 rpm for 10 minutes to remove red blood cells. The veins were removed from the HBSS and incubated in 0.53 mmol/L 0.05% trypsin/EDTA and collagenase 100 U/mL for 10 minutes each at 37°C. The veins were discarded and the collagenase solution centrifuged to pellet the CJVECs. The CJVECs were suspended in complete EC media containing 5 ng/mL FGF-1 and 5 U/mL heparin and plated on a fibronectin-coated T-25 culture flask (2.5 μg/cm^2). Cells were incubated at 37°C and 5% CO_2, and the media changed every 2 to 3 days. The cells were immunofluorescently stained by using factor VIII antibodies to confirm their endothelial identity. Only cell populations of greater than 95% purity were used for the angiogenesis assay, and CJVECs were used in passages 1 to 3.

Methocel preparation

Methocel was prepared as described previously [10]. Methylcellulose reagent (1.2 g) was autoclaved in a 250-mL beaker at 120°C for 20 minutes. The autoclaved powder was dissolved in 50 mL of preheated (60°C) endothelial basal medium, stirred at 60°C for 20 minutes, and then 50 mL of endothelial basal medium at room temperature was added. The solution was mixed for 2 hours at 4°C and then centrifuged for 2 hours at 4,000 rpm. Methocel was the supernatant after centrifugation.

Angiogenesis assay

The angiogenesis assay was a combination of models developed by Xue and Greisler [11] and Kroff et al [10]. Briefly, CJVECs were trypsinized and resuspended in complete media without FGF-1; 20,000 CJVECs were suspended in 40-μL drops containing 20% methocel solution in complete EC media. The drops were incubated in suspension culture at 37°C for 48 hours on a Petri dish lined with parafilm. Cell aggregates formed, and the media was aspirated carefully by using a dissecting microscope. The cell aggregates were then embedded in fibrin glue (FG). FG was prepared by using 2.5 mg/mL fibrinogen in M199 at pH of 7.8. The fibrinogen solution polymerized immediately after addition of 0.32 U/mL thrombin.

The cell aggregates were sandwiched between two 150-μL FG solutions that were polymerized on a nylon mesh ring, allowing easy manipulation of the gels. Once the FG gels polymerized, the disks were transferred into 24 wells plates and complete growth medium was added containing 100 KIU/mL aprotinin. Aprotinin was used in order to slow the degradation of the FG to support 3-dimensional network formation. The aggregates were stimulated with 0, 1, 10, or 100 ng/mL of FGF-1 with 5 U/mL of heparin. The disks were cultured at 37°C and 5% CO_2 for a period of 23 days. The assay media and cytokines were changed every 2 days.

Quantitative and statistical analysis

The EC aggregates form capillary networks, which were quantified daily for 21 days as described previously [11]. The aggregates were imaged by using a Zeiss Axiovert 200M microscope (Carl Zeiss MicroImaging, Inc, Thornwood, NY) daily, and a grid was used to allow