Microvascular Research 90 (2013) 40–47

Contents lists available at ScienceDirect

Microvascular Research

journal homepage: www.elsevier.com/locate/ymvre

Angiogenic endothelial cell invasion into fibrin is stimulated by proliferating smooth muscle cells☆

Areck A. Ucuzian [a,b,*], Dominick V. Bufalino [a], Yonggang Pang [c], Howard P. Greisler [a,b,c]

[a] Department of Surgery, Loyola University Medical Center, 2160 S 1st Ave, Maywood, IL 60153, USA
[b] Department of Cell Biology, Neurobiology, and Anatomy, Loyola Medical School, 2160 S 1st Ave, Maywood, IL 60153, USA
[c] Surgery and Research Services, Hines VA Hospital, 2100 S 5th Ave, Hines, IL 60616-3793, USA

ARTICLE INFO

Article history:
Accepted 28 June 2013
Available online 22 July 2013

ABSTRACT

These studies aimed to determine the effect of smooth muscle cells (SMCs) on angiogenic behavior of endothelial cells (ECs) within fibrin hydrogels, an extracellular matrix (ECM) commonly used in tissue engineering. We developed a 3-D, fibrin-based co-culture assay of angiogenesis consisting of aggregates of SMCs with ECs seeded onto the aggregates' surface. Using digital fluorescence micrography, EC matrix invasion was quantified by average length of sprouts (ALS) and density of sprout formation (DSF). We demonstrated that ECs and SMCs co-invade into the ECM in close proximity to one another. ECs that were co-cultured with SMCs demonstrated increased invasion compared to ECs that were cultured alone at all time points. At Day 19, the ALS of ECs in co-culture was 327 +/− 58 μm versus 70 +/− 11 μm of ECs cultured alone ($p = .01$). The DSF of co-cultured ECs was also significantly greater than that of ECs cultured alone ($p = .007$ on Day 19). This appeared to be a function of both increased EC invasion as well as improved persistence of EC sprout networks. At 7 days, ECs in co-culture with proliferation-inhibited SMCs previously treated with Mitomycin-C (MMC) demonstrated significantly attenuated sprouting compared to ECs co-cultured with SMCs that were untreated with MMC (82 +/− 14 μm versus 205 +/− 32 μm; $p < .05$). In assays in which multiple co-culture aggregates were cultured within a single hydrogel, we observed directional invasion of sprouts preferentially towards the other aggregates within the hydrogel. In co-culture assays without early EC/SMC contact, the ALS of ECs cultured in the presence of SMCs was significantly greater than those cultured in the absence of SMCs by Day 3 (320 +/− 21 μm versus 187 +/− 16 μm; $p < .005$). We conclude that SMCs augment EC matrix invasion into 3-D fibrin hydrogels, at least in part resulting from SMC proliferative and invasive activities. Directed invasion between co-culture aggregates and augmented angiogenesis in the absence of early contact suggests a paracrine mechanism for the observed results.

Introduction

Pericytes, derived from vascular mesenchymal cells such as smooth muscle cells (SMCs) and adventitial fibroblasts, surround the endothelium of capillaries and contribute to the later maturation and stabilization stages of angiogenesis. Direct contact via gap junctions, adhesion plaques, and adherens junctions, as well as indirect paracrine mechanisms between endothelial cells (ECs) and pericytes, allow for significant interactions which impact EC differentiation, migration, proliferation, and quiescence (Gerhardt and Betsholtz, 2003; Gerhardt et al., 2000; Rucker et al., 2000; Tillet et al., 2005). In addition, pericytes and their precursors appear to have a significant role in mediating the early events of active angiogenesis (Brey et al., 2004; Kunz-Schughart et al., 2006; Ozerdem et al., 2001; Ozerdem et al., 2002). Proteins including VEGF and deposited extracellular matrix (ECM) proteins such as fibronectin secreted by SMCs and fibroblasts in vitro have been demonstrated to elicit angiogenic activities such as increased plasminogen activity, proliferation, motility, and cordlike structure formation in co-cultured ECs (Chon et al., 1997; Davie et al., 2006; Dietrich and Lelkes, 2006; Kale et al., 2005; Kuzuya et al., 1995; Montesano et al., 1993). Evidence that the local extracellular environment may regulate paracrine interactions between ECs and pericytes is provided by the observed endothelin-1-mediated activation of EC mitogenesis by fibroblasts, (Davie et al., 2006) and the production of Ang-2 and VEGF in mesangial cells (renal pericytes) under hypoxic conditions (Yuan et al., 2000). In vivo observations of pericytes "leading" angiogenic sprouts and lining vascular tubes that lack ECs suggest a more direct role for pericytes as guiding cells during angiogenesis (Nehls et al., 1992; Ozerdem and Stallcup, 2003). These data are consistent with observations that under hypoxic stimulation, VEGF-secreting perivascular cells appear to be the first vascular cells to invade the *corpus luteum* parenchyma during ovulatory angiogenesis (Reynolds et al.,

☆ NIH R01-HL41272 (HPG), AHA Award 0815674G(AAU) and the Department of Veterans Affairs (HPG).

* Corresponding author at: Division of Vascular Surgery, UPMC Heart & Vascular Institute, 200 Lothrop Street, A-1017, Pittsburgh, PA 15213. Fax: +1 412 291 1669.
E-mail addresses: aucuzian@gmail.com (A.A. Ucuzian), dbufalino@lumc.edu (D.V. Bufalino), pangyonggang@gmail.com (Y. Pang), Hgreisl@lumc.edu (H.P. Greisler).

0026-2862/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.mvr.2013.06.012

2000). Thus, while the contribution of pericytes to the later stabilization and maturation of neocapillaries is well established, the likelihood that they promote angiogenic induction given the proper pro-angiogenic environmental context suggests a broader role for these cells which warrants further study (Nehls and Drenckhahn, 1995).

In tissue engineering, the need for microvasculatures to supply nutrients and oxygen for constructed organs such as tissue-engineered blood vessels (TEBVs) is thought to be critical. Specifically, the utilization of mesenchymal cells such as SMCs and fibroblasts to construct the wall of a tissue-engineered blood vessel may alter the angiogenic processes within the wall of the vessel that precede TEBV vasa vasorum development. The purpose of these studies is to determine how SMCs affect the angiogenic behavior of endothelial cells within fibrin hydrogels, an extracellular matrix frequently used in tissue engineering, and a common component to hydrogel-based TEBVs. We hypothesize that SMCs will promote angiogenic activity by promoting ingrowth into a 3-D fibrin matrix. We will also test if contact between SMCs and ECs is necessary for promotion of angiogenic activity or inhibitory of SMC-mediated effects on EC angiogenic activity. We designed three unique in vitro co-culture models of angiogenesis in order to address these questions.

Materials and methods

Materials

Chemicals, biological reagents, and experimental supplies were obtained as follows: collagenase (Invitrogen); human thrombin (American Red Cross; Rockville, MD); Mitomycin C, L-ascorbic acid, methylcellulose, fibrinogen, transferrin, insulin, anti-α-actin antibody and aprotinin, anti-VWF antibody, PKH-27, PKH67 (Sigma Chemical Co.; St. Louis, MO); bovine lung heparin (Upjohn; Kalamazoo, MI); 0.05% trypsin/EDTA, HBSS, M199, DMEM, L-nonessential amino acids, sodium pyruvate, penicillin, streptomycin, DMEM-F12 (Gibco, Grand Island, NY); fetal bovine serum (FBS) (Hyclone, Logan, UY); 100 mm and 60 mm Petri dishes (Fisher Scientific; Pittsburgh, PA); round bottom 96-well plates (Greiner Bio-one; North Carolina); 96-well polystyrene plastic plates (Beckton Dickinson; Lincoln Park, NJ); woven nylon mesh rings (ID = 7.5 mm, OD = 13 mm) (Sefar America Inc.; Kansas City, MO); Parafilm M (American National Can, Greenwich, CT); fetal bovine serum (FBS) (Hyclone, Logan, UY); 24-well plates and tissue culture flasks, Costar Transwell polystyrene plates (Corning Costar Corp; Cambridge, MA).

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) and were approved by our institutional IACUC.

Cell isolation

SMCs and ECs were harvested from adult mongrel canine carotid arteries and external jugular veins respectively as per our previously published protocols (Brewster et al., 2004; Kang et al., 1995). EC identity was confirmed using immunofluorescent dual staining with anti-vWF and anti-α-actin antibodies. Only EC exhibiting 95% positive vWF staining and 2% or less α-actin staining were used. ECs were used within passages 1 to 5. Primary SMCs migrating from the carotid artery explants were used in all experiments. SMC identity was confirmed by immunofluorescent staining with anti-α-actin antibodies, and only cultures exhibiting 95% positive staining were used in the assays. Individual carotid artery explants were used as the source of cells for assays no more than 3 times.

Mitomycin C treatment

In experiments using Mitomycin C (MMC) treatment to inhibit cell proliferation, 500,000 SMCs were plated onto 60 mm Petri dishes and treated with 10 μg/mL of MMC in SMC growth media containing DMEM, 10% FBS, PCN/Strep (100 U/mL), Gentamicin (.05 μg/mL), Amphotericin (.25 μg/mL), non-essential amino acids, MEN, and sodium pyruvate for one hour prior to use in experiments as described in previously published methods (Ucuzian et al.). Preliminary experiments and previously published data demonstrated viability of cells as well as significant inhibition of SMC proliferation demonstrated by reduced thymidine incorporation and of matrix invasion when treated with this protocol (Ucuzian et al.). SMCs that were concurrently plated in SMC growth media, but not treated with MMC, served as controls.

Angiogenesis assays

Co-culture angiogenesis assay #1: EC/SMC contact model

SMCs were fluorescently labeled green with PKH-67 (10 μM) and ECs were fluorescently labeled red with PKH-26 (10 μM) as per manufacturer instructions. PKH is a cell membrane label which incorporates into the plasma membrane of cells and is divided equally into subsequent progeny cells (Fox et al., 1999). An aggregating solution consisting of angiogenesis assay media (M199, 10% FBS, PCN/Strep (100 U/mL), Amphotericin (0.25 μg/mL), aprotinin (100 KIU/mL), heparan sulfate (5 U/mL)), 2250 green fluorescently labeled SMCs, and a 20% Methocel solution (Uriel et al., 2006) was added into each well of a round bottom 96-well plate and incubated at 37 °C for 24 h until cell aggregates formed. After 24 h, 350 red fluorescently labeled ECs were added to each well and allowed to seed the surface of the SMC aggregate. These cell numbers were based on preliminary experiments which optimized EC seeding to form a co-culture aggregate with an SMC core and as close to an EC monolayer as possible. In experiments which tested the effects of inhibiting SMC proliferation on EC matrix invasion, ECs were seeded onto aggregates of SMCs which were either treated or untreated with MMC as described above. To ensure that only SMCs were treated with MMC, the SMCs were washed with PBS 3 times prior to use in co-culture assays. The co-culture aggregates of ECs and SMCs were then embedded between two layers of fibrin that were polymerized such that the aggregates were completely surrounded by the hydrogel. Fibrin hydrogels were constructed by preparing a fibrinogen solution consisting of fibrinogen (2.5 mg/mL), M199, and heparin (5 U/mL). The solution was polymerized by the addition of thrombin (0.32 U/mL). Once completely polymerized, the disks were cultured in angiogenesis assay media (M199, 10% FBS, PCN/Strep (100 U/mL), Amphotericin (0.25 μg/mL), aprotinin (100 KIU/mL), heparan sulfate (5 U/mL)) at 37 °C (Fig. 1). After a period of time, SMCs and ECs invade from the central aggregate into the surrounding hydrogel as described in previous publications (Fig. 2) (Brewster et al., 2010; Ucuzian et al.; Xue and Greisler, 2002). Aggregates of 2500 fluorescently labeled ECs and no SMCs served as the control group.

Digital images were taken daily for up to 19 days at a magnification of 4× and quantified using a Zeiss Axiovert 200 M microscope (Carl Zeiss, Inc., Oberkochen, Germany) and Axiovision software. The digital photographs were aligned with a grid evenly divided into 36 radial intervals using Adobe Photoshop for quantification using both TRITC and FITC filters. For each assay, the average length of sprouts (ALS) was defined as the average of the furthest distance of invasion from the central aggregate on each grid line. A secondary data point quantifying the density of sprout formation (DSF) was also measured by calculating the number of grid lines with crossing sprouts/36 total grid lines (Fig. 2). The presence of cellular debris and discontinuity of previously continuous chains of invading cells is indicative of cell death (Uriel et al., 2006). For this reason, sprouting was quantified by measuring only out to the final point of a continuous chain of cells. Notably, invasion into an