HYPOXIA/REOXYGENATION ENHANCES TUBE FORMATION OF CULTURED HUMAN MICROVASCULAR ENDOTHELIAL CELLS: THE ROLE OF REACTIVE OXYGEN SPECIES

Peter I. Lelkes, Kenneth A. Hahn, Soverin Karmiol, and Donald H. Schmidt

Laboratory of Cell Biology, Department of Medicine, University of Wisconsin Medical School, Milwaukee Clinical Campus.
$*: Section of Cardiology, Department of Medicine, University of Wisconsin Medical School, Milwaukee Clinical Campus.
*: current address: BioWhittaker, Inc. 8830 Biggs Ford Road, Walkersville, MD 21793.

INTRODUCTION

Angiogenesis, the generation of new blood vessels, is a ubiquitous process which is tightly regulated in normal physiological situations. The cellular and molecular mechanisms controlling the initiation and termination of the angiogenic process are only partially known (Folkman and Klagsbrun, 1987; Folkman and Shing, 1992; Maragoudakis, 1994; Ferrara, 1996; Montesano et al., 1996; Pepper et al., 1996). The pathophysiology of many diseases involves uncontrolled growth of new blood vessels, prompting the search for therapeutically effective inhibitors of angiogenesis (Maragoudakis, Sarmonika, and Panoutsacopoulou, 1988; Folkman and Ingber, 1992; Fotsis et al., 1993; D'Amato et al., 1994; O'Reilly et al., 1994; Polverini, 1994; Chen et al., 1995; Gradishar, 1997; O'Reilly et al., 1997). Conversely, in other clinical settings, promotion of neovascularization is desirable, e.g. after myocardial infarction and/or in peripheral blood vessel occlusion, thus calling for appropriate stimulators of "therapeutic" angiogenesis (Höckel et al., 1993; Isner et al., 1995).

Numerous prior studies have firmly established that hypoxia enhances angiogenesis in vivo, presumably by upregulating the expression/production of angiogenic growth factors, such as vascular endothelial cell growth factor (VEGF) in non-endothelial cells, e.g. in smooth muscle cells or fibroblasts, concomitant with the upregulation of cognate VEGF receptors, such as KDR/flk, and flt on endothelial cells (Shweiki et al., 1992; Millauer et al., 1993; Seetharam et al., 1995; Brogi et al., 1996; Takagi et al., 1996; Tian, McKnight, and Russell,
1997). Recently, Shono and coworkers have shown that chronic exposure to hypoxia (at least 3 days) enhances the formation of capillaries in an in vitro model of angiogenesis (collagen gel), presumably by up-regulating VEGF, and/or its cognate receptor(s), activating PKC-dependent signaling, or inducing the secretion of angiogenic cytokines (Shono et al., 1996).

In contrast to these lengthy studies in the collagen gel system, culturing endothelial cells on a complex, laminin-rich reconstituted basement membrane (Matrigel) extracted from the Englebreth-Holm Swarm (EHS) tumor, will rapidly yield a capillary-like network of tubular structures, termed herein tube-formation or tubular morphogenesis (Kubota et al., 1988; Grant et al., 1991). The Matrigel system represents a particular in vitro angiogenesis system, which adequately models certain stages of the angiogenic cascade, such as EC migration, elongation and assembly of tubes (Baatout, 1997). In spite of these limitations, the Matrigel system has been widely used, to considerable success, to elucidate some of the basic mechanisms of in vitro angiogenesis. Recently cultured endothelial cells on Matrigel under experimental conditions of hypoxia/reoxygenation similar to those encountered in clinical situations of transient ischemia and observed, enhanced tubular morphogenesis (Hahn et al., 1995a). We hypothesized that exposure of HMVEC to hypoxia/reoxygenation might lead to the intracellular generation of reactive oxygen species (ROS) and that endogenous ROS might directly and rapidly affect the angiogenic properties of the cells cultured on a permissive 3-D extracellular matrix, such as Matrigel (Hahn et al., 1995b). In this communication we will detail some of the salient experimental findings and provide evidence for the involvement of ROS in the initiation of the angiogenic cascade.

MATERIALS AND METHODS

1. Cell Culture: Human dermal microvascular endothelial cells (from Clonetics Corporation, San Diego, CA) were grown in an optimized cell culture medium (MCDB 131 supplemented with 10 ng/ml EGF, 1 µg/ml hydrocortisone, 30 µg/ml bovine brain extract, 10 µg/ml heparin, 50 µg/ml gentamicin, 50 ng/ml amphotericin B and 5 % fetal calf serum, as previously described (Manolopoulos, Samet, and Leikes, 1995; Silverman et al., 1996; Kanda et al., 1998). The endothelial nature of these cells has been previously established by a number of criteria (immunostaining for vWF, PECAM-1 and uptake of acetylated LDL). Based on previous experience, most of the phenotypic traits of these cells, including their capability to rapidly form tubes on Matrigel (in vitro angiogenesis), were maintained through the 9th passage, corresponding to 25-30 population doublings (Leikes et al., 1994; Leikes et al., 1996). Accordingly, only cells from the 4th through the 9th passage were used for these studies.

2. In Vitro Assay for Tube Formation on Matrigel: Matrigel was prepared in house from EHS tumor (generously provided by Dr. Hynda K. Kleinman, NIDR, NIH, Bethesda MD) according to published procedures (Grant et al., 1991; Haralabopoulos et al., 1994). To generate a solid, three-dimensional Matrigel base, 12 well cell culture plates (surface area = 3.5 cm²/well, from Costar) were coated with 100 µl/cm² ice cold Matrigel and incubated overnight at 37°C.