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An assay for macrophage-mediated regulation of endothelial cell proliferation

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Abstract

We have developed an assay that quantifies the potential of macrophages to regulate proliferation of endothelial cells. We show that young mice macrophages can be distinguished from old mice macrophages by their ability to inhibit vascular endothelial cell proliferation. While young mice macrophages robustly inhibit proliferation, old mice macrophages fail to do so and actually promote the proliferation of endothelial cells. In this report, we outline a technique that directly assesses the effect of macrophages on modulation of endothelial cell proliferation. This assay will help us in understanding the mechanisms of macrophage function in several disease states characterized by abnormal angiogenesis including cancers, angiogenic eye disease and atherosclerotic heart disease.

Keywords: Angiogenesis; Endothelial; Immunity; Innate; Macrophage; Vascular

Introduction

Angiogenesis and neo-vascularization is subject to various levels of control, including regulation of endothelial cell (EC) proliferation. EC proliferation can be regulated by soluble mediators like VEGF, BDNF and angiopoietin and it can also be suppressed by cytotoxicity (Adamis et al., 1996; Witmer et al., 2003). Cell-mediated cytotoxicity is a major effector pathway of immune protection against intracellular pathogens or tumors and a fundamental mechanism to maintain homeostasis of the immune system. The cytotoxic response is mediated by different cell types and involves several different mechanisms of death induction (Berke, 1991; Russell and Ley, 2002). Cytotoxic cells secrete granules containing various lytic enzymes, which then act on the target cells (Shiver et al., 1992). Macrophages play an important role in host immune defense against bacterial infections, tumors and disease states characterized by abnormal angiogenesis (Apte et al., 2006; Blood and Zetter, 1990; Dace and Apte, 2008; Gordon, 1998; Kelly et al., 2007). An important component of inflammatory reactions and subsequent repair and remodeling processes is angiogenesis or neo-vascularization – the formation of new capillaries from preexisting blood vessels (Folkman and Shing, 1992). Macrophages have been shown to be pro- and anti-angiogenic depending on the type of stimulation given, alternatively activated macrophages are pro-angiogenic and classically activated macrophages act as anti-angiogenic cells (Ruszczak et al., 1990; Rutherford et al., 1993; Schreiber et al., 1986; Sullivan et al., 1983). EC proliferation and migration leads to the development of new blood vessels, a process that is important in cancers and angiogenic eye diseases that impair vision.

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We wanted to develop an assay that would quantitatively determine the direct effect of macrophages on vascular EC proliferation.

Materials and methods

Animals

C57BL6 mice were purchased from Jackson Laboratories; they were either used as young mice (<3 months old) or old mice (derived by aging them in the barrier animal facility at Washington University to >18 months of age). Human micro vascular endothelial cells (HMVEC) were purchased from Lonza (Walkersville, MD formerly Cambrex) and cultured in specific media purchased from Lonza. Thymidine was purchased from Amersham (Piscataway, New Jersey). All work was carried out in accordance with Association for Research in Vision and Ophthalmology (ARVO) guidelines for the *Use of Animals in Ophthalmic and Vision Research*.

Proliferation assay

Effector cell preparation

Young B6 (<3 months) and old (>18 months) B6 mice were injected intraperitoneally with 3% thioglycollate. Five days later macrophages were harvested with sterile PBS from the peritoneum of these mice and cultured in RPMI-1640 + 10% FCS overnight. Macrophages were then washed with RPMI-1640 to remove non-adherent cells. Cells were stimulated with LPS (100 ng/ml) for 12 h. Macrophages were washed with RPMI + 10% FCS at 1200 rpm for 8 min (Fig. 1).

Target cell preparation

HMVEC in log phase were washed with PBS and incubated with trypsin/EDTA at 37 °C. Trypsin/EDTA was neutralized with trypsin neutralizing solution (TNS) from Lonza. Cells were then spun down at 1000 rpm for 8 min. Cells were counted and plated in 96-well round bottom plate at a concentration of 5×10^4 for adherence to the plate.

Co-culture

Macrophages from effector cell preparation were resuspended in EGM2V (Lonza, Walkersville, MD), a HMVEC-specific medium. Medium was removed from HMVEC culture and macrophages were added at various target:effector ratios to HMVEC (1:1 EC/M, 1:10 EC/M and 1:25 EC/M). Cultures were continued for an additional 12 h, then the tritiated thymidine (40 μCi/ml) (TRA61-GE Health Care, Piscataway, NJ) was added to the cultures for 12–18 h. Plate was

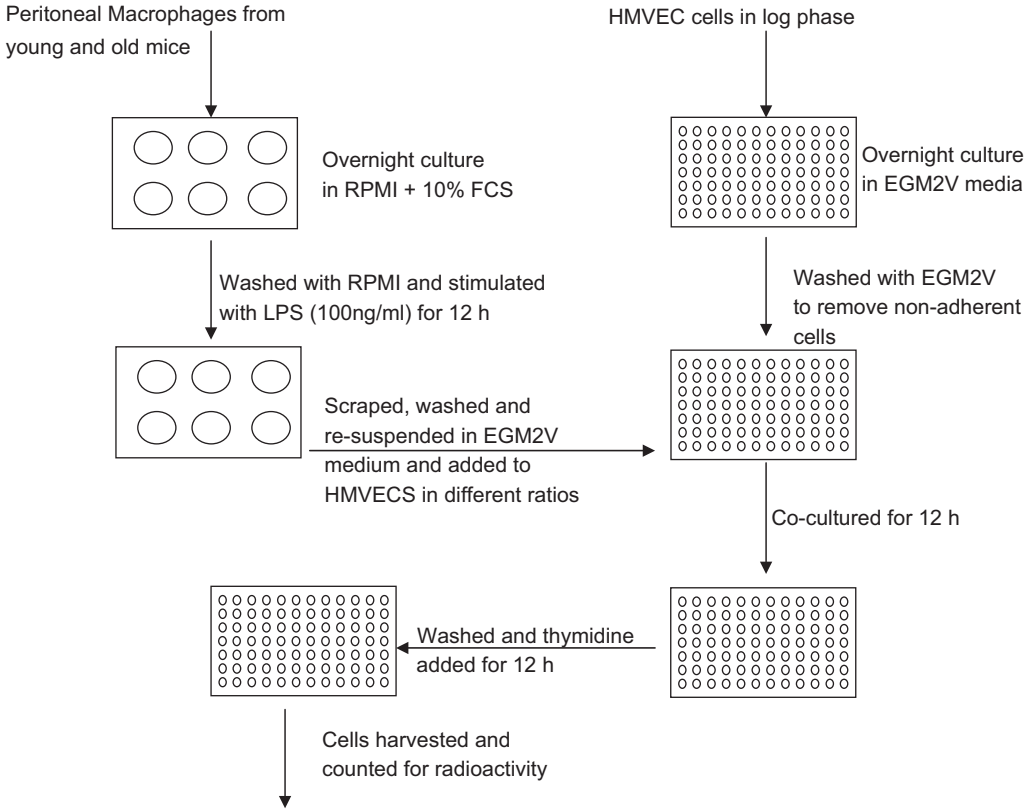

Fig. 1. Schematic representation of vascular endothelial proliferation assay.